The biological and therapeutic significance of tumour necrosis

Identification and characterisation of viable cells from the necrotic core of multicellular tumour spheroids provides evidence of a new micro-environmental niche that has biological and therapeutic significance.

Charlotte Louise Evans

submitted for the degree
of Doctor of Philosophy

Institute of Cancer Therapeutics

University of Bradford

2014

Abstract

Charlotte Louise Evans

The biological and therapeutic significance of tumour necrosis

Identification and characterisation of viable cells from the necrotic core of multicellular tumour spheroids provides evidence of a new micro-environmental niche that has biological and therapeutic significance.

Key Words: Necrosis, Multicellular Tumour Spheroids, Tumour Microenvironment, Autophagy, Metabolism, Chemoresistance.

Tumour necrosis has long been associated with poor prognosis and reduced survival in cancer. Hypotheses to explain this include the idea that as aggressive tumours tend to grow rapidly, they outgrow their blood supply leading to areas of hypoxia and subsequently necrosis. However whilst this and similar hypotheses have been put forward to explain the association, the biological significance of the cells which make up necrotic tissue has been largely ignored. This stems from the belief that because a tumour is more aggressive and fast growing it develops areas of necrosis, rather than, the tumour is more aggressive because it contains areas of necrosis. Which came first like the egg and chicken is yet to be determined, however to date most research has only considered the possibility of the former. Viable cells were found in the necrotic core of Multicellular Tumour Spheroids. When examined these cells were found to be different to the original cell line in terms of proliferation, migration, and chemosensitivity. A proteomic analysis showed that these phenotypical changes were accompanied by changes in a large number of proteins within the cells, some of which could be potential therapeutic targets. Furthermore this has led to a new hypothesis for tumour necrosis

and its association with poor prognosis. Necrotic tissue provides a microenvironemental niche for cells with increased survival capabilities. Protected from many chemotherapeutics by their non-proliferative status once conditions improve these cells can return to proliferation and repopulate the tumour with an increasingly aggressive population of cells.

Acknowledgments

I would like to thank Yorkshire Cancer Research for sponsoring my studentship, my supervisors Roger Phillips and Chris Sutton for their guidance and support throughout the course of my degree as well as everyone at the Institute of Cancer Therapeutics.

Special thanks must go to Andrei Mardaryev, Patricia Cooper, and Sadr Al Shaheed whose expertise and advice made this project possible.

Finally thanks must go to my family and friends for their unending support and confidence that I would succeed.

Table of Contents

Introduction

1 The Cancer Problem

Cancer is a disease caused by the uncontrolled division of abnormal cells. In 2012 an estimated 14.1 million people were diagnosed with cancer worldwide with the four most common cancers being lung, breast, bowel and prostate. In the same year the number of cancer related deaths reached 8.2 million with the majority of deaths as a result of lung, liver, stomach or bowel cancer.[1] Over the past 4 decades there have been significant developments not only in the treatment of cancer but also in our understanding of the pathogenesis of cancer. In 2000 the landmark paper by Hannahan and Weinberg [2] characterised cancer as the acquisition of 6 different traits or 'hallmarks' that are essential for carcinogenesis. These hallmarks include self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. More recently in 2011 a further paper was published which added two emerging hallmarks, deregulated cellular energetics and avoiding immune destruction and two new enabling characteristics, genome instability and mutation, and tumour promoting inflammation to the list.[3]

The treatment of cancer involves a team of multidisciplinary specialists including surgeons, radiotherapists and oncologists who administer chemotherapeutic agents. The history of chemotherapy goes back to the effects of mustard gas used during World War I on white cell counts and the subsequent application of nitrogen mustard as a treatment for haematological malignancies.[4, 5] While significant advances have been made in the treatment of certain cancers such as lymphoma, leukaemia, and testicular carcinomas are now treatable, the response of lung, colorectal, oesophageal, pancreatic and prostate cancers remains poor and is often only palliative.[6] Toxicity to normal tissue and the emergence of drug resistant cells are two of the key reasons why classical cytotoxic

drugs fail to reduce mortality rates[7] and the need for new therapies that selectively target tumours as opposed to normal tissue and circumvent drug resistance has been recognised for many years. As the molecular basis for cancer continues to be unravelled, novel therapeutics that target key hallmarks of cancer are emerging. One particularly successful example is the Philadelphia chromosome discovered in chronic myelogenous leukaemia[8] which led to the development of Imatinib targeted against the fusion protein Bcr-Abl.[9] Additional advances have been made in several areas and a new generation of targeted therapeutics have emerged.[10] These developments underpin the perceived wisdom that understanding the biology behind cancers is the real driving force leading to the development of new targeted therapies for cancer treatment. As more targeted therapeutics progress into the clinic, it has become increasingly clear that they also have their problems. New mechanisms of resistance have emerged and despite initial good responses to therapy, overall survival is frequently extended for periods of months not years. This phenomena has been attributed to the fact that tumours are genetically heterogenous and new 'strains' of aggressive cells emerge following the suppression of specific pathways.[11] Understanding how tumours escape chemotherapy and re-emerge as an aggressive tumour is essential if long-term remissions are to be obtained.

On this theme, the hypoxic tumour microenvironment is a known cause of resistance to therapy and is a marker of poor prognosis. Hypoxia is a phenomenum whereby a tissue is deprived of adequate levels of oxygen leading to biochemical changes within the cells. Hypoxia is known to promote the emergence of a more aggressive phenotype via several mechanisms including selection of p53 mutant cells that don't undergo apoptosis under hypoxic conditions.[12] In addition to hypoxia, solid tumours are known to contain regions of necrosis and the extent of necrosis has been shown to be a marker

of poor prognosis in several cancer types. The mechanistic basis underpinning this relationship is not known. This thesis will focus on the tumour microenvironment with a specific focus on tumour necrosis as a 'niche' where cells can still survive very hostile conditions leading to resistance to therapy and the emergence of a resistant and increasingly aggressive population of cells. Initially however, it would be pertinent to review the literature focusing on the biological origins of tumour necrosis and the current understanding of how necrosis may lead to poor prognosis.

2 Necrosis

2.1 Types of Cell Death

Cell death can generally be divided into two distinct types, apoptosis, a type of programmed cell death which involves a distinct combination of signalling pathways and has characteristic morphological changes and necrosis which has traditionally been considered as an uncontrolled and passive form of cell death. Where apoptosis can be likened to a form of cell suicide which requires energy in the form of ATP, necrosis is more cataclysmic, accidental and uncontrollable cell death which results in the release of the cellular contents into the extracellular space.[13] Necrosis is also characterised by certain morphological changes such as disruption and breakdown of the plasma membrane and induction of inflammation in the surrounding tissue as a result of the release of the cellular contents into the extracellular space. Karyolyis occurs which entails DNA being degraded leading to the chromatin of the nucleus fading, making the dark blue nuclei seen in H&E staining less visible. As the chromatin condenses the nucleus itself starts to shrink, a phenomenon known as pyknosis. Then the nucleus begins to fragment and the nuclear material is distributed throughout the cytoplasm in a process called karyorrhexis. Necrosis occurs due to a catastrophic energy shortage

where the levels of cellular ATP become incompatible with the cell's survival. This energy shortage can be the result of a number of external stimuli including toxins, pathogen infection, physical and heat damage and a critical lack of oxygen or nutrients.[14]

There are many lesser known forms of cell death other than apoptosis and necrosis. Cells can die via mitotic catastrophe which tends to be defined by cell death which occurs as a result of aberrant mitosis and occurring during or shortly after mitosis itself.[15, 16] Entosis is described as a non-apoptotic cell death as a result of detachment from the ECM which leads to the unusual phenomenon of invasion of one cell into another. This cell internalisation leads to one cell containing another still living cell and can result in either degradation of the internalised cell termed entosis or release of the cell.[17] Panthanatos is used to describe a particular subtype of cell death which involved the poly ADP ribose polymerase (PARP) DNA damage response enzymes, in particular PARP1. Whilst normally PARP1 is involved in the repair of single strand DNA breaks in order to ensure genomic homestasis in response to minor DNA damage. However overactivation of PARP1 can cause depletion of NAD+ and ATP.[18, 19]

2.2 Morphological patterns of tissue necrosis

There are three types of tissue necrosis, coagulative, colliquative and caseous, each type has a different macroscopic presentation (see Figure 1).[20] Coagulative necrosis presents as firm tissue, almost as if it has been cooked due to the production of a gel-like substance, in this case the architecture of the tissue is maintained. Denaturation of protein within the cell leads to coagulation as the albumin within the cells becomes firm and gel like.[21] Colliquative necrosis also known as liquefactive necrosis is more liquid-like in presentation, as digestion of the dead cells transforms the tissue into a viscous

mass. Whilst this commonly occurs as a result of a bacterial or fungal infection, hypoxic infarcts in the brain can cause this type of necrosis due to the tissues lack of connective tissue and high levels of digestive enzymes. [22] Caseous necrosis appears like a combination of colliquative and coagulative, with a structure more often described in layman's language as cottage cheese. The tissue appears white and friable as the dead cells are only partly digested leaving behind granular particles (Figure 1).[23] In the case of necrosis found in tumour tissue this presents as coagulative necrosis.[24]

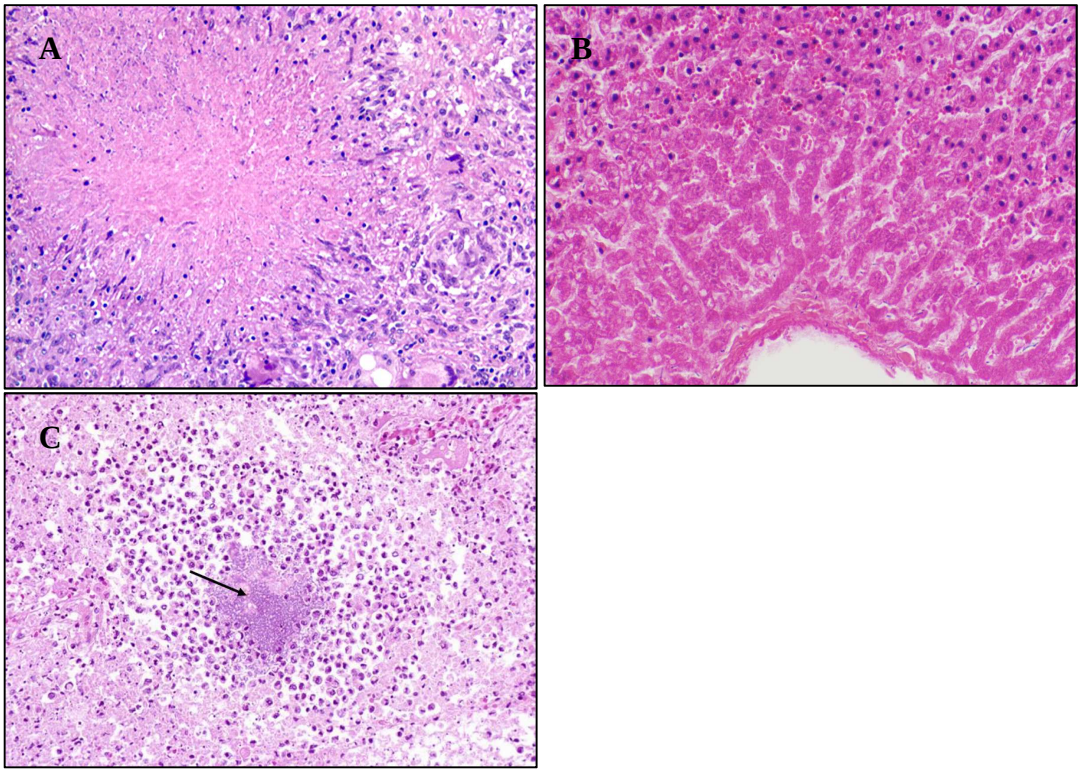


Figure 1. Types of Necrosis. Panel A Caseous Necrosis in the lymph nodes, Panel B Coagulative necrosis in the liver, Panel C Liquifactive necrosis in the lung the small area of necrosis is denoted by an arrow. [25]

2.3 Necrosis: a type of programmed cell death?

Recent evidence has shown that in some cases necrosis may be more regulated than previously thought. It is thought it may function as a backup mechanism of cell death which is avoided where possible due to the consequences of the process on the

surrounding tissue, but can be activated in cases of emergency.[26] It has been discovered that in certain cases where necrosis is not triggered by very extreme conditions the process has been documented as a regulated event. Multiple signalling pathways involving death receptors,[27] the mitochondria[28] and certain kinase cascades[29] have been showed to play a part in necrosis. Indeed by inhibiting certain molecules in these pathways a cell can be influenced towards either apoptosis or necrosis.[30, 31, 32] Anti apoptotic molecules such as Bcl-2/Bcl-x proteins and heat shock proteins have been found to prevent not just apoptosis but in some cases necrosis as well.[33, 34] In some cases caspases were required for the initiation of necrotic process.[35, 36] Inhibition of caspases such as caspase-3 and caspase-8 inhibited not only apoptosis but also necrosis.[37, 38, 39] Furthermore necrosis has been found to be involved in various physiological processes throughout the body. Renewal of the cells lining the small intestine has been shown to involve necrosis as well as apoptosis,[40] and similar findings have been seen in the large intestines.[41] During oogenesis, necrosis has been discovered to play a role in follicular development,[42] and natural selection in the immune system specifically the activation dependant death of T-cells is both caspase independent and has necrotic morphology.[43]

Necroptosis is the term used to describe regulated necrosis which involves distinct molecular signalling including death receptor signalling, caspase inhibition and RIP1 (receptor interaction protein 1) and/or RIP3 activation.[44] Necroptosis has been implicated in a number of different disease types including myocardial infarction, stroke and inflammatory bowel disease.[45, 46] The process has been found to be stimulated by multiple triggers such as alkylating DNA damage[47] and excitotoxins.[48] The kinase RIP1 is known to play an integral part in the initiation of necroptosis as necrostatin-1 an inhibitor of necroptosis has been to exert its anti necroptosis effect through the

inhibition of RIP1 sernine/threonine kinase activity.[49] A number of death receptors have been identified which can stimulate necroptosis including TNFα, FasL and Trail.[50]

Whilst some of the stimulators of necroptosis have been uncovered the executioner proteins further downstream remain more elusive. Proposals have been made for the involvement of Reactive oxygen species (ROS) based upon the evidence that the ROS scavenger, butylated hydroxyanisole, inhibited TNFα-induced necrotic cell.[51] Autophagy has also been implicated in the execution of necroptosis due to the observation that autophagosomes are often present in cells known to be undergoing necroptosis. Further research into autophagy's role has shown that knockdown of beclin1 and Atg7, two autophagy related genes, was sufficient to inhibit necroptosis in murine fibrosarcoma cell line.[52] However this has yet to be demonstrated in human cell lines. Necroptosis may activate autophagy, hence making autophagy a response to necroptosis as opposed to part of the process itself.[53] Whilst research is still ongoing in order to fully understand the process and implications of necroptosis in disease, neurostatin could be a potential new treatment for diseases involving inflammation as a result of necroptosis.

2.4 Necrosis as a pathological indicator of prognosis in cancer

In solid tumours areas of necrotic tissue are found where the tumour has outgrown its current blood supply. The location of necrosis is thought to correspond with intra tumour hypoxia.[54] Increased tumour necrosis has long been associated with increased aggressiveness of the tumour.[55] It has been noted that a reduction in necrosis is seen in tumours in elderly patients compared to younger patients. Specifically this has been found in breast cancer where tumours in younger patients (<39yrs) were found to show increased tumour necrosis compared to tumours found in older patients. This is

suggestive of necrosis being correlated with increased tumour aggressiveness as tumours in younger patients are significantly more aggressive than those seen in older patients.[56] More recently in colorectal cancer, the presence of necrosis was found to be associated with poor differentiation, large tumour size, advanced stage and venous invasion.[57] Further studies have found that necrosis in colorectal tumours is linked to poor survival.[58]

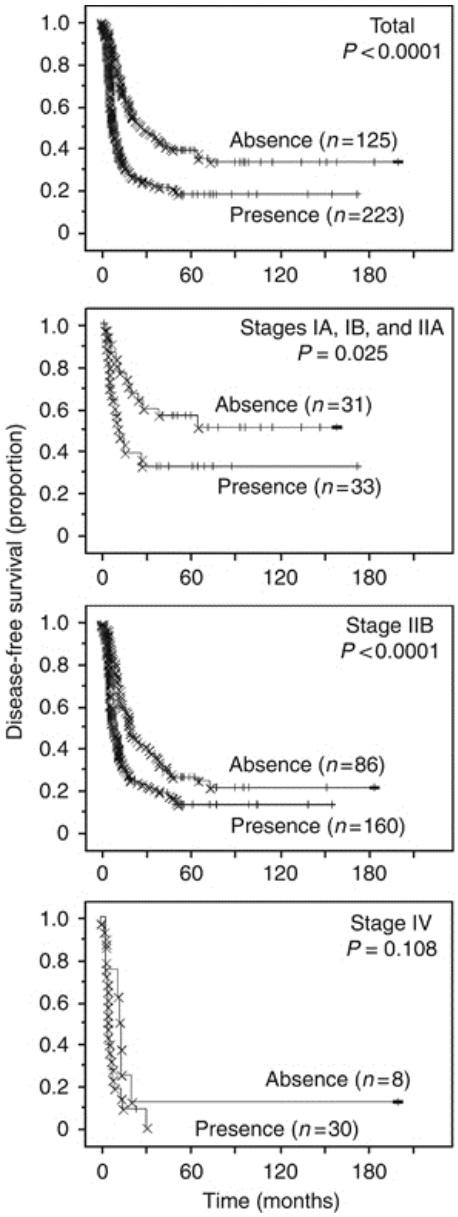


Figure 2. Kaplan-Meier Survival Plots. Detailing the differences in survival time between pancreatic cancer patients with tumour necrosis and without at different stages of progression. As the disease progresses the effect of necrosis upon survival time lessens.[59]

This data has been backed up in a number of other solid tumour types. Necrosis has been linked to poor prognosis in renal cell carcinoma, where it was also associated with other prognostic indicators like lymph node disease, metastases, high grade and tumour size.[60] What this study also showed however was that only necrosis in primary tumours was an indicator of poor prognosis and reduced overall survival. When the patient sample was separated into two groups, those with localised disease and those with metastatic, necrosis was found to be a predictor of prognosis only in those with localised disease. While it is not known exactly why this is theories have been put forward to explain it. Such as, tumours with

necrosis have been shown to be more aggressive therefore it is not surprising that these aggressive tumours are capable of metastasising, and the development of metastases is clearly an indicator of poor prognosis. As opposed to patients who already have metastatic deposits, their prognosis is already determined far more by the presence of metastases rather than any necrosis in the primary tumour. The same phenomenon has also been document in prostate cancer (see Figure 2). Necrosis has also been recently found to be of prognostic value in upper urinary track transitional cell carcinoma,[61] gastrointestinal tract tumours[62] and Ewing's sarcoma amongst others.[63] Also in non small cell carcinoma extensive tumour necrosis was found to correlate with an aggressive tumour phenotype[64] and poor prognosis.[65] However one large study showed that tumour necrosis in NSCLC (non small cell lung cancer) was not correlated with micro vessel density, [66] leading to the question if necrosis does not reflect the angiogenic capacity of the tumour then how and why is it correlated with tumour aggression and poor prognosis?

2.5 Investigating the link between necrosis and tumour aggression

Whether the high levels of necrosis directly cause the tumour to be more aggressive, or it is an indirect association between rapidly growing, aggressive tumours that outgrow the blood supply leading to necrosis, is unclear.[67]

There are many theories to explain the link between necrosis and tumour aggressiveness. Such as the tendency to see more necrosis in highly angiogenic tumours, which at first seems counterintuitive, however angiogenesis allows the tumour to rapidly proliferate, and this can lead to areas of ischemia within the rapidly growing tumour as proliferation quickly surpasses the new angiogenic growth. Furthermore, tumour vasculature is unlike normal vasculature found in the rest of the body, as it is poorly organised and leaky, this can lead to not all areas of the tumour being adequately

supplied. This theory is supported by the fact that high levels of necrosis are associated with larger more vascular tumours.[68]

Areas of inflammation and necrosis are also known to be conducive for increased genetic mutations which exacerbate genetic instability and promote the emergence of more aggressive tumours via the acquisition of additional mutations. In tumours low nutrient availability can lead to oxidative stress and the build-up of damaged proteins and organelles. This would normally stimulate p62 which would bind to the aggregates and target them for destruction via the autophagic pathway. However in cells with deficient autophagy this response is absent and both p62 and its damaged cargo build up until a cytotoxic response is stimulated. This can involve the stimulation of DNA damage responses, changes in gene expression and increased loss/gain of chromosomes. All this can work to increase the frequency of DNA mutations and hence stimulate further tumour progression and potentially the emergence of a more aggressive tumour.

Ultimately whilst it has been observed that tumour necrosis is indicative of poor prognosis, the exact mechanisms behind this association are not yet understood. By examining the necrotic tissue itself and the cells which it is composed of, a better understanding could be formulated. Determining whether all tumour cells in areas of necrosis are indeed dead or whether some cells possess the necessary biochemical machinery to survive is an important first step in investigating the link between necrosis and tumour aggression.

Investigating the biological mechanisms behind tumour necrosis will help to provide evidence towards the hypothesis that Tumour necrosis is associated with poor prognosis for patients as it provides a microenvironmental niche for the development of an increasingly aggressive, metastatic and treatment resistance population of cells. And

that the emergence of this population explains the association between tumour necrosis and patient prognosis.

Before discussing the concept further it would be pertinent to first of all review the origins of tumour necrosis and the cellular responses to severe microenvironmental conditions.

3 The Tumour Microenvironment

3.1 Heterogeneous nature of tumours

As mentioned previously one of the emerging hallmarks of cancer is deregulated cellular energetics which involves the capacity of the cell to reprogram its metabolism in order to survive and proliferate in changing tumour microenvironments. The tumour microenvironment has been gaining interest in the role it plays in tumour biology. The tumour microenvironment can be broadly described separately in terms of its two compartments, the stromal microenvironment and the hypoxic microenvironment. Normally the stroma acts as a barrier against tumour formation, however the presence of transformed cancer cells can cause changes in the stromal cells which leads to an increasingly tumour promoting environment.[69] Cells which make up the stromal compartment include those which associated with the tumour vasculature namely endothelial cells, fibroblasts, macrophages, and immune cells including lymphocytes. The extracellular matrix is also a crucial part of the tumour microenvironment. Pericytes which wrap around the endothelial cells making up the tumour vasculature are known to be involved in paracrine signalling to maintain endothelial cell homeostasis. Pharmacological targeting of these cells causes loss of coverage leading to vascular instability, loss of integrity and function, importantly normal non tumour associated pericytes are not sensitive to pharmacological targeting, making this selective to tumour

vasculature.[70, 71] Tumour promoting immune cells include, macrophages, mast cells, neutrophils, and B and T lymphocytes. Research has discovered that these cells can express and release various signalling molecules into the stroma which carry out their tumour promoting actions.[72, 73] Fibroblasts are also found in solid tumours, they are often the preponderant cell population making up the stroma. Cancer associated fibroblasts (CAFs) have been found to support the surrounding tumour cells through paracrine signalling and a parasitic-host metabolic interaction whereby the tumour cells stimulate the CAFs to undergo autophagy in order to provide recycled nutrients for the tumour cells.[74]

The hypoxic tumour microenvironment is another important consideration as the majority of tumours contain regions of low oxygen concentration also known as hypoxia. The pathogenesis behind this physiological microenvironment can be explained in part by the tumour vasculature.

In order for a tumour to grow past a certain size, it needs to develop a vascular supply. The type of vessels formed and their structure leads to the generation of a tumour microenvironment which serves to help the tumour resist treatment.[75] Tumour vasculature tends to be disorganised and leaky with blind ends, and arterio-venous shunts, which fails to supply all cells within the tumour consistently. This coupled with a poor lymphatic drainage system, changes in extracellular matrix (ECM) composition and contraction of the interstitial space mediated by fibroblasts, causes high interstitial fluid pressure (IFP).[76] High IFP directs blood to the periphery of the tumour and away from the centre. Also another contributing factor is the high metabolic activity of the tumour cells meaning oxygen consumption within the tumour is high.[77] All this leads to a tumour microenvironment where nutrient and oxygen availability is reduced, and cells become starved of nutrients and oxygen, often leading to the formation of necrosis. The

presence of tumour necrosis has been found to be correlated with tumour aggression, with the more aggressive tumours showing higher levels of necrosis. The reason for this association is yet to be elucidated but various theories will be discussed in detail later.

Hypoxia can have many effects upon a tumour's biology; it can lead to the selection of cells with genotypes more suited for the low oxygen conditions[78] and changes in cell signalling in processes governing apoptosis[79] and autophagy[80] and cell metabolism.[81] Hypoxia can also promote certain physiological processes such as angiogenesis,[82] epithelial mesenchymal transition,[83] invasion and metastases[84] and as such can play a role in drug resistance through a variety of different mechanisms.[85]

In order to take advantage of this characteristic of the tumour microenvironment hypoxia related signalling pathways, and in particular hypoxia-inducible factor (HIF) which controls the transcription of a number of hypoxia related genes, have been investigated as targets.[86] As well as the physiological reduction in oxygen levels which has led to the development of hypoxia activated pro drugs. These bio reductive pro drugs are inactive until they reach the hypoxic environment of the tumour where they are reduced to their active form and can then exert they effect upon the cancer cells. Two examples of hypoxia activated pro drugs are tirapamazine which induces single and double strand breaks[87] and AQ4N a topoisomerase II inhibitor[88] which are both selectively activated in the hypoxic tumour environment. The advantage of this type of drug is that they are less toxic to normal tissues due to the added selectively.[89] Hypoxic cells are known to be more resistant to certain chemotherapeutics and radiotherapy[90] making this microenvironment problematic when treating cancer, while at the same time giving rise to new targets.

The acquisition of chemotherapeutic resistance is a sign of active tumour evolution and the influence of environment pressures. The interactions between the tumours cells and the tumour microenvironment, and how this affects resistance needs to be more fully understood in order to discover new therapeutic targets, and explain the failure of previously investigated targets. The targeting of numerous cellular compartments may lead the way to overcoming therapeutic resistance and achieving improved patient responses.

3.2 The metastatic microenvironment

Tumour hypoxia has long been linked with increased tumour aggression and metastatic spread.[91, 92] In order for cells to successfully metastasise they must first be able to penetrate through the basement membrane and then undergo the process of intravasation to gain access to the circulatory system. Once in the circulation, if the cells can survive, they are transported to a distant site where they must then go through extravasation. Whilst studies have shown that the frequency of arrival of metastatic cells at new sites is quite high, only a small number manage to thrive in their new environment.[93] In order for a new cell to grow in its new environment it must receive the correct signals from the ECM, if the new microenvironment is more conducive to quiescence then the cell may not be able to proliferate and form micro metastases. [94] The biology of this can be explained by the before mentioned 'seed and soil' theory which explains why certain cancers tend to metastasise to certain organs such as prostate to bone, colorectal to liver and breast to brain. In order for tumour cells to grow in a new environment the new environment must be conducive to growth of that particular cell. Which means that microenvironments in different tissue are more appropriate for the growth of different cancer cells. Evidence behind this theory shows that circulating cancer cells with certain complements of cell surface receptors tend to implant in new microenvironments which

have reciprocal receptors/ligands. Chemokines in particular have been shown to be involved in the process. Cancer cells express specific chemokine receptors and they have been found to metastasise to sites which express the corresponding ligand. This was seen in breast cancer where metastasising cells were found to express the two chemokine receptors CXCR4 and CCR7, the corresponding ligands CXCL12 and CCL21 were found to be produced in the lung and lymph nodes, two common sites for breast cancer metastasis. Furthermore blocking the receptors with antibodies was shown to reduce the frequency of metastasis.[95] This highlights the idea that to help prevent metastasis the molecular microenvironment of the organs where metastasis often occurs needs to be investigated carefully for new targets which could prevent the survival or growth of new cancer cells there.

3.3 Therapeutic implications of the tumour microenvironment

Cancer cells from different tumour types vary highly in their genetic complement of mutations; however changes in biology caused by the tumour microenvironment are hopefully more common to a wider variety of tumour types with similar microenvironments. This would suggest that approaches to target the microenvironment could be more transferable between different cancers. The first step in doing so would be to identify the individual molecular targets in the microenvironments of different tumour types. This been done for a number of different tumour types so far.[96, 97]

Over time the understanding of the biological and therapeutic implications of the tumour microenvironment has increased greatly and the full impact of its contributions to not only primary tumour formation but tumour progression and invasion and metastasis, are finally being appreciated. This increased understanding of the differential pathophysiology in tumour tissue compared to normal tissue has lead and continues to lead to the discovery of new molecular targets.

4 Multicellular Tumour Spheroids (MCTS) as a model of the Tumour Microenvironment.

Cancer's heterogenesis nature makes it important to choose the right model in which to study *in vitro*. Specifically the heterogeneity of the physical environments found within solid tumours. Regional differences in pH, oxygen concentrations and nutrient availability impose differing selective pressures upon tumour cells in different areas of the tumour. Two dimensional monolayers are the standard model for studying both the biology of cancer and the effects of chemotherapeutics *in vitro*. While they have undeniable benefits such as their reproducibility, simplicity to set up, and viability of cells in culture, the use of 3D models has recently undergone a resurgence in interest. One of the reasons for this is that solid tumours like breast cancer and colorectal cancer are not two dimensional but three and as such are physically different to cancer cells grown as a monolayer. Whilst every cell in a monolayer has equal access to nutrients and oxygen, in solid tumours only the cells in the viable rim have unobstructed access. Once inside the tumour the cells are at the mercy of diffusion gradients and a constantly changing and unreliable vasculature. Therefore there are distinct populations of cells which have adapted to living in different microenvironments depending on their proximity to tumour or host vasculature and as such are both phenotypically heterogeneous and can respond differently to chemotherapeutics.

Multicellular tumour spheroids (MCTS) have been around since the 1970s where they were mostly used as an *in vitro* three dimensional model for studying radiotherapy and chemotherapy in solid tumours.[98, 99] Since then their value as a model for studying the tumour microenvironment has been realised, and they have been used to study a variety of different phenomenon such as hypoxia[100] and tumour cell metabolism[101] in a less artificial way compared to work done in monolayers. MCTS are one of the most

common forms of 3D culture and are particularly popular as they can be grown in large numbers. They are physically symmetrical, and this symmetry extends to the physiological characteristics like cell-cell adhesion, nutrient gradients, and areas of hypoxia and necrosis. They most represent avascular tumours and early micrometastasis.

4.1 Advantages of MCTS as an in vitro model for studying the physiological microenvironment of tumours

One of the main benefits of MCTS is that they are three dimensional and are more representative of an actual tumour than a monolayer of human cancer cells grown in a flask. This is due to the fact that the cells within a MCTS, unlike monolayers, are subject to diffusion gradients of oxygen and nutrients leading to the formation of a necrotic core similar to solid tumours (see Figure 3). In a MCTS the outer cells are able to proliferate due to the close proximity of the source of nutrients and oxygen, whilst the innermost cells cannot. This selective pressure can lead to different populations of cells within the same spheroid (Figure 4). Cells which have different phenotypes respond differently to chemotherapy, specifically chemotherapeutics which target proliferating cells.

As the distance from the surface of the MCTS hence from the source of oxygen and nutrients increases the concentrations of both decrease whilst concentrations of waste productions increase (see Figure 3). At a critical distance often ~500µM for many cancer cell lines[102, 103] the cells can no longer survive the conditions. Similarly MCTS will only grow to a certain size (~1000µm in diameter for many cell lines) before their growth stalls.[104] Originally it was believed that oxygen concentrations dictated the thickness of the viable rim and once oxygen levels dropped below a critical point the cells became necrotic. It was discovered however that oxygen is not the sole contributor

to this process, cells can become necrotic within MCTSs at a variety of different oxygen concentrations, with other factors such as glucose levels being implicated.[105]

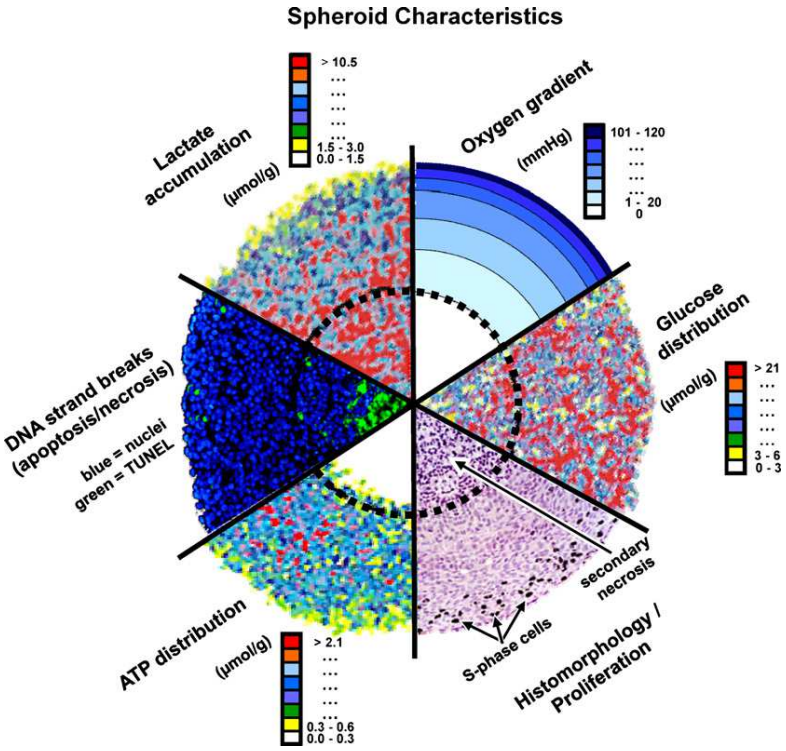


Figure 3. Diagrammatic representation of the different gradients which exist within MCTSs.[106]

The cells which reside at the innermost part of the spheroid become quiescent due to nutrient and oxygen deprivation and eventually die, mostly through necrosis. These differential growth kinetics resemble those of tumours grown in vivo, particularly micro metastases prior to angiogenesis. As the mass of cells increases in size the proportion of cells actively proliferating is reduced, while the proportion of quiescent cells is increased.[107] Similarly in patients the tumour cells which are located near a blood vessel are actively proliferating but cells which are not located near a blood vessel exist in a non-proliferative dormant stage. Whilst much effort has been put into targeting and killing the proliferating tumour cells, the dormant cells by their very nature are resistant to many chemotherapeutic drugs as they target actively proliferating cells.[108] These

dormant cells could contribute to relapse once the proliferating cells have been depleted through chemotherapy yet there has been little progress in targeting them.[109, 110] One reason for the lack of progress could be the difficulty in finding an appropriate in vitro model to study this phenomenon in.

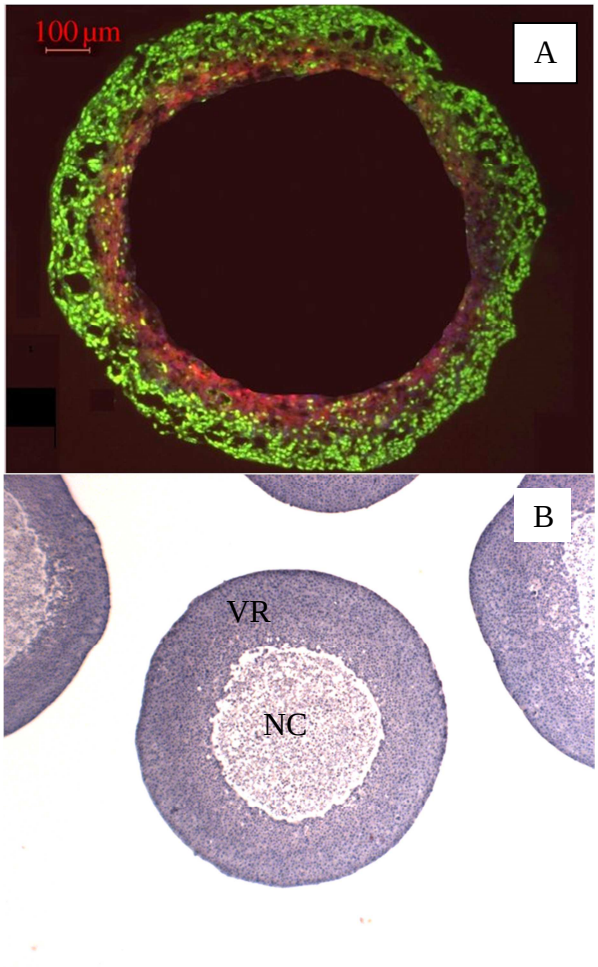


Figure 4. Hypoxia and Necrosis in DLD-1 MCTS. Panel A hypoxia in a DLD-1 MCTS. Green represents Ki67 positive proliferating cells, red represents pimidozole staining representing the hypoxic population of cells.[111] Panel B Shows a HT-29 MCTS (for further HT-29 spheroid H&E staining see Chapter 2 results) stained with haematoxylin to detect the nucleus and eosin to highlight the cytoplasm, showing the thick viable rim (VR) and central necrotic core (NC).

4.2 Co-culture MCTS

MCTS can also support the co-culture of different cells types, enabling stromal cells types such as fibroblasts to be grown with cancer cells. This model, which more closely resembles the cellular heterogeneity that is seen in *in vivo* tumours can be used to look at tumour-stromal interactions, which given recent findings appear to be increasingly important when looking at the consequences of the tumour microenvironment.[112]

4.3 Culturing Spheroids

There are a variety of techniques that can be used to culture spheroids. One of the most widely used involves spinner flasks which allow the cultivation of large numbers of spheroids at the same time. The theory behind growing spheroids involves preventing the attachment of the cells to any surface. Spinner flasks prevent cells from attaching to the surface of the flask by keeping the media constantly moving using a stirrer rod. In order to grow, the cells then have to attach to each other, and it is the process of inducing cells to attach to each other and grow that is fundamental for the formation of spheroids.

4.4 MCTS as an *in vitro* model for solid tumours.

Whilst spheroids were initially used for testing the response of different cell populations to radiotherapy, since then they have been used as a model for studying the effects of chemotherapeutics, in particular the penetration of chemotherapeutics into the spheroids. Similar to quantifying the effects of radiotherapy, spheroid diameter is taken to establish spheroid growth delay as a measure of drug efficacy. Proliferation can also be measured using autoradiography on spheroids treated with H-thymidine, histologically using BrdU antibodies or antibodies against proliferation markers such as Ki-67, or on single cell populations via flow cytometry. Though the three dimensional structure of spheroids makes it innately impossible to study single cells from the

spheroid *in situ*, by freezing or fixing and then sectioning spheroids different cell populations can be characterised. Multicellular mediated resistance has been seen in spheroids in response to certain drugs including alkylating agents like cyclosphosphamide[113] and cisplatin.[114] This is similar to the 'contact effect' seen in spheroids in response to radiotherapy whereby cells in a spheroid are more resistant than those grown in a monolayer. Although the reason behind this has not been fully established it is believed to be due to a combination of factors. Gap junction 'reciprocity' which is essentially the ability of cells to provide nutritional support to neighbouring sick or starving cells, is thought to be involved,[115] along with changes in repair-related gene expression as a result of cell shape and modifications in chromatin packaging which affects DNA repair.[116] Multicellular mediated resistance is believed to be caused by the same factors involved in the 'contact effect.'[117] The fact that there are such differences in the response of cells grown in monolayers compared to spheroids only highlights the importance of three dimensional cell culture as an intermediate step between classical *in vitro* monolayers and *in vivo* testing.

4.5 Cell Sorting

The ability to sort the different cellular populations within a MCTS allows for the individual characterisation of these cells, and can highlight the effects of known microenvironmental changes on the cells. Cells from MCTS can be sorted using different methods including serial trypsinisation where the enzyme tryspin is used to digest cells from the outside of the MCTS one layer at a time. Another method involves using non-lethal nucleic dyes such as Hoechst 33342 to stain the intact spheroids. Once stained it is possible to use FACS to separate the cells into different populations based on their level of fluorescence. Cells located on the outer edges of the spheroid would be

most strongly stained as the dye takes time to diffuse through the cells and penetrate the very centre of the spheroid.[118, 119]

By using fluorescent associated cell sorting (FACS) it is possible to sort the cells in the spheroids into many different populations, based on their distance from the viable rim. These different populations can then be individually examined for relative numbers of necrotic, apoptotic autophagic and quiescent cells. The populations can also be assayed to compare the cells' viability and aggressive characteristics such as the ability to invade and metastasise. The response of the different cellular populations within the MCTS to chemotherapeutics and a measure of drug penetration can also be achieved by examining the sorted populations.[120]

5 Cellular responses to the hypoxic tumour microenvironment

All cells not only cancer cells are able to adapt up to a point to changes in their local environment. Such mechanisms that can be used by the cells; particularly cancer cells in response to stress, nutrient deprivation and hypoxia, include autophagy, tumour dormancy, senescence, apoptosis, epithelial mesenchymal transition and many more. The tumour specific responses to the tumour microenvironment which can increase the survival and progression of the cancer are discussed in the following section.

5.1 Autophagy

Cells can adapt biochemically to different micro-environmental conditions and knowledge of these changes has potential implications for the development of novel therapeutics. The selective pressure leads to the development of a subpopulation of cells more adapted to their environment. Adaptations can include processes such as autophagy. The word autophagy in Greek literally translates to 'self eating' and is a biological process which has been known for decades but has only recently come to the

forefront of several areas of disease research. There are three types of autophagy, macroautophagy, microautophagy and chaperone mediated autophagy (CMA).[121] Both micro and macroautophagy involve sequestering portions of the cytoplasm, via the formation of an autophagosome de novo, in microphagy the substrates in the form of individual proteins, are taken up straight into the lysosome by invagination of the membrane. CMA requires no membrane rearrangements but instead relies on a molecular chaperone Hsp90 and a lysosome-associated membrane protein LAMP-2a to transport proteins across the membrane of the lysosome.[122]

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved process of protein degradation which occurs in all eukaryotic cells.[123, 124] It is a highly regulated process that works to maintain cellular homeostasis under conditions of intra or extra-cellular stress such as nutrient deprivation by recycling long lived proteins and damaged organelles back to single amino acids to aid cell survival. Autophagy can also be stimulated under conditions such as oxidative stress, accumulation of damaged organelles and pathogen infection. Its stimulation can lead to either adaption to conditions and survival or death via a process known as programmed cell death II (autophagic cell death).[125, 126] In the case of bacterial infection, bacteria can be selectively targeted for degradation within the autophagolysosomes,[127] or alternatively the bacteria can trigger autophagic cell death.[128] Autophagy occurs under many different pathological conditions, including cancer and neurodegenerative diseases such as Parkinson's, Huntington's and Alzheimer's. However whether autophagy acts in a protective or detrimental manner under these conditions is unclear. The following sections examine the process of autophagy in more depth with a particular focus on the different steps involved in the process, it's role in cancer and different stimuli for induction.

5.2 Autophagy Signalling

In response to stimuli, a double membrane structure named the isolation membrane, forms in the cytoplasm. The origin of which is currently unknown. The membrane elongates as it sequesters portions of the cytosol, where upon the edges of the isolation membrane fuse together to form a complete autophagosome. The autophagosome then traffics its cargo to a lysosome, and fuses with it to create an autophagolysosomes. Once fusion has occurred the lysosomal hydrolases degrade the sequestered contents which are then released back into the cytosol and recycled to provide fuel for protein synthesis (Figure 5). [129]

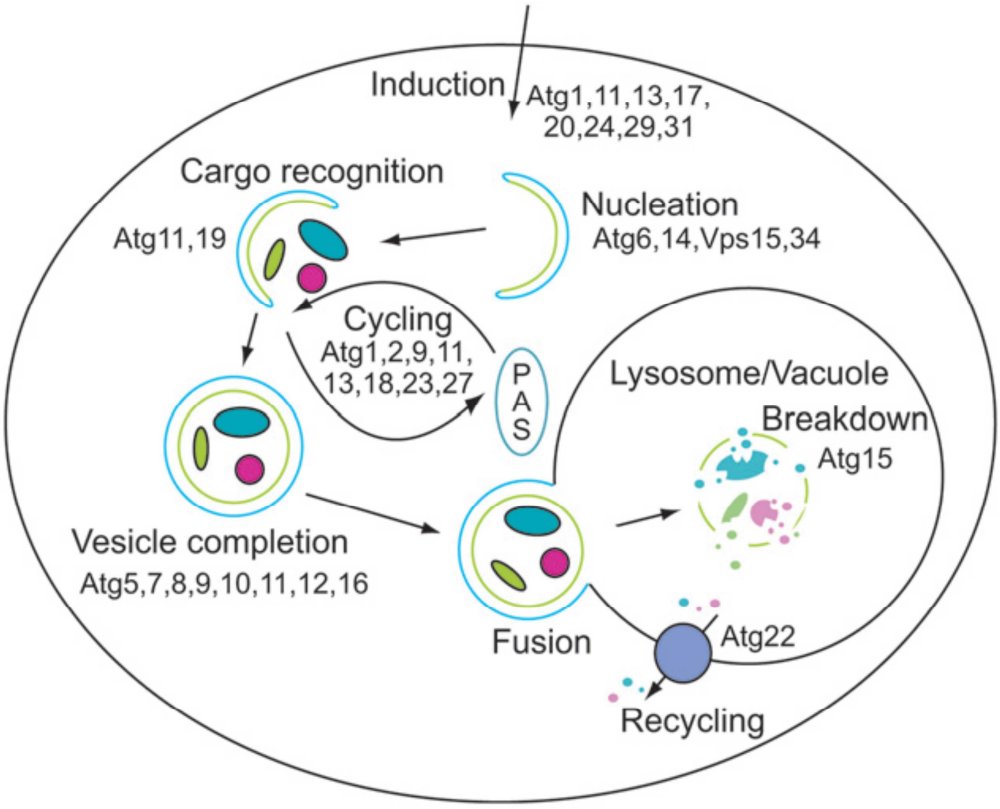


Figure 5. Diagram representing the molecular mechanism of autophagy and the genes involved in each of the steps as found through studies in yeast. The autophagic process is split into several steps; induction, vesicle nucleation, cargo recognition, vesicle completion, fusion with the lysosome, vesicle breakdown, and nutrient recycling. The Atg proteins involved in each of the steps are shown. The phagophore assembly site (PAS) which is believed to be the location for the assembly of the phagophore is shown.[130]

Autophagy is essentially a process for recycling old or misfolded proteins and damaged organelles in order to provide a source of energy for starving cells, and also to provide a measure of quality control by ridding the cells of damaged components. This is particularly useful in post mitotic cells where there is no option to dilute these damaged proteins via cell division.[131] The digested proteins and organelles provide both starting material for the production of new cellular structures as well as energy when the new amino acids are fed into the tricarboxylic acid (TCA) cycle to produce ATP.[132]

Over the past years the machinery involved in autophagy has been determined using yeast as a model organism. More than 30 autophagy-related genes (ATGs) have been discovered so far in yeast, many with mammalian equivalents. [133] The autophagy pathway has been characterised into a series of separate steps including induction, cargo recognition and selection, vesicle nucleation, vesicle expansion and completion, autophagosome-vacuole fusion and breakdown of cargo (see Figure 5), of which similar processes have been identified in mammalian cells. So far, the function of many Atgs is now understood.

There are different subtypes of autophagy, which differ depending on the stimulus for autophagy induction. Lack of nutrients tends to stimulate a type of autophagy where inhibition of mTOR leads to a reduction in the association of p62 with the autophagosome membrane, and the autophagosomes are more likely to contain large elements of free cytosol. The autophagosomes themselves, in starvation-induced autophagy are thought to be derived from mitochondria.[134, 135] In contrast, a separate subtype of autophagy is thought to be stimulated by stress (e.g. oxidative) or the build-up of ubiquitinated aggregates, and differs in that p62 is associated with the autophagosome membrane in the initiation stage. Autophagosomes stimulated by such conditions are more likely to contain ubiquitinated cargo.[136]

5.3 Stimuli for Autophagy Induction

In most cells basal levels of autophagy are quite low under normal conditions.[137] Autophagy can be induced by several stimuli, of which the most common and well known is lack of nutrient availability. This is detected via AMPK (adenosine monophosphate-activated protein kinase) which senses the low AMP-ATP ratio and becomes activated through the action of LKB1 kinase (Serine/Threonine Kinase 1).[138] Activated AMPK then goes on to phosphorylate Raptor, one of the subunits of mTORC1 (mammalian target of rapamycin complex 1, a regulator of protein synthesis and cellular growth and proliferation pathways) to cause induction of autophagy. AMPK also acts to activate TSC (tuberous sclerosis complex) which leads to the inhibition of Rheb and subsequent inhibition of the mTORC1 complex, which prevents the mTORC1 mediated phosphorylation of Ser757 on Ulk1. This allows activated AMPK to phosphorylate Ulk1 on Ser 777 and 317 which then renders the complex active and induces autophagy (Figure 6). [139]

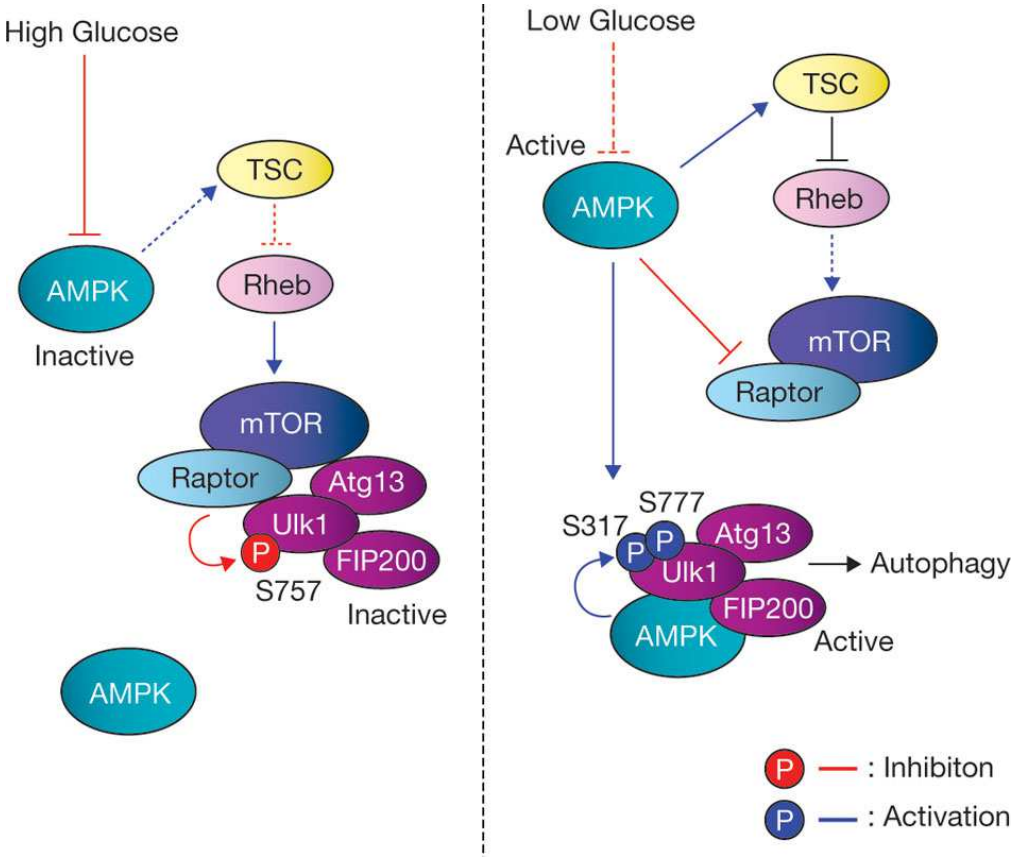


Figure 6. Pathway for autophagy regulation via AMPK under conditions of high and low glucose availability.[140]

5.4 Hypoxia, oxidative stress and autophagy

Autophagy can also be induced under conditions of mitochondrial stress such as a build up of reactive oxygen species (ROS).[141] Under starvation conditions ROS produced from the mitochondria have been reported to alter the activity of Atg4 a cysteine protease autophagy protein. In this state Atg4 still 'primed' LC3 by removing amino acids from the C terminal end of the protein but no longer carried out its delipidating actions further downstream by removing phosphatidylethanolamine from LC3 (known as LC3-II). This prevents LC3-II from being recycled back to LC3-I, which ensures the maintenance of high levels of LC3-II ready to fuse with the membranes of the phagophore and autophagosome.[142] This method of induction links autophagy to another process characteristic of the tumour microenvironment, hypoxia. Autophagy has

been shown to be induced in hypoxic cells that characterise solid tumours.' [143] This process has been found in some cases to be dependent upon HIF-1 , specifically HIF-1 dependant expression of BNIP3. [144] This induction acts to protect the cell under unfavourable conditions by allowing them to adapt to the hypoxic environment and also by reducing the cell's apoptotic potential.[145] In other cases however induction of autophagy in response to hypoxia has been found to be HIF-1 independent. This has been found in cases of severe hypoxia (<0.1% oxygen) often in the presence of nutrient deprivation. Implicated in this induction is AMPK. The induction of autophagy under these conditions has been found to be associated with autophagic cell death and is considered a failed attempt at adaptation. [146] In cells being treated with certain chemotherapeutics autophagy has been shown to have a protective effect on the cells, not just allowing them to survive low oxygen conditions but also in response to chemotherapeutics by reducing their apoptotic potential. For instance in one study hepatocellular carcinoma cells were shown to be more resistant to chemotherapeutics under hypoxia compared to normoxia; however this difference was abolished by the addition of autophagy inhibitors to the hypoxic cells.[147]

Hence hypoxia induced autophagy and the mechanisms governing this can have both pro survival and pro death effects depending on the nature of the stimulus.[148, 149]

5.5 Autophagy and Cancer: The Autophagy Paradox

Autophagy has been linked to both tumour suppression and tumourigenesis as many proteins involved in autophagy are also known oncogenes and tumour suppressors. The process and function of autophagy within cancer is however a complicated matter. In some instances cancer cells undergo autophagy as a defence mechanism against nutrient shortages, and in tumours with limited blood supply autophagy works to help those cells not sufficiently fed by diffusion from blood vessel to survive (Figure 7). In these

circumstances if autophagy is inhibited this can then lead to relatively selective tumour cell death.[150] Furthermore autophagy has also been found to correlate with poor outcome in pancreatic ductal adenocarcinoma,[151] which is thought to be due to dependence of pancreatic ductal adenocarcinoma cells on autophagy for growth.[152] Recently several trials have started which are looking into the effects of an autophagy inhibitor (hydrochloroquine) in combination with the standard treatment regime for a variety of solid tumours (breast, prostate, lung) plus multiple myeloma.[153]

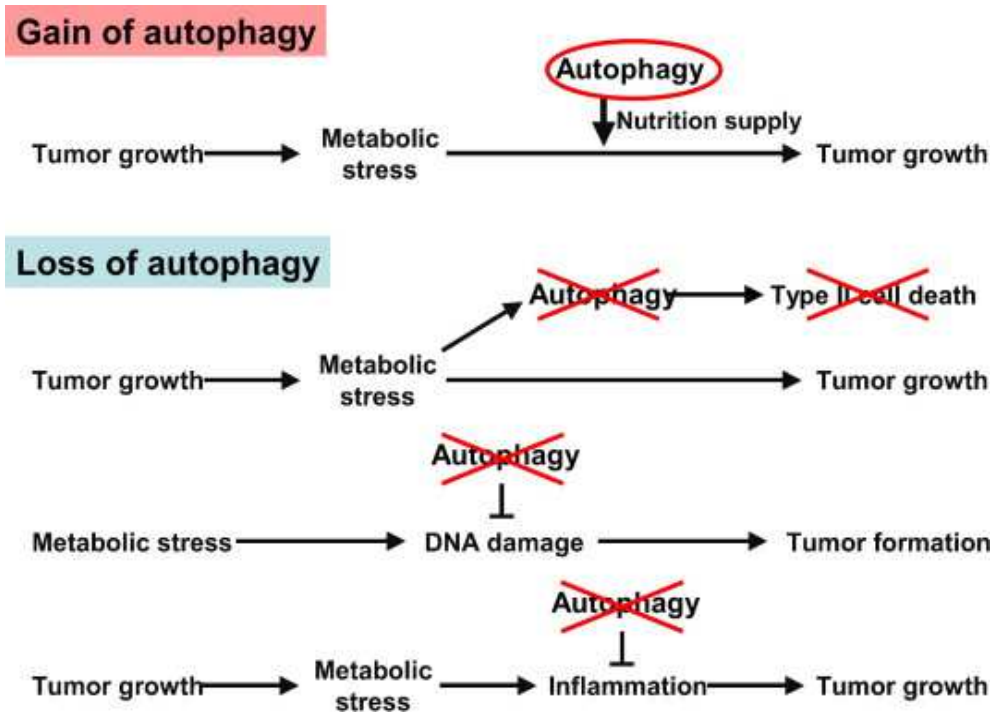


Figure 7. The differing roles of autophagy in tumour formation and growth. Autophagy can be both induced and inhibited in order to help tumours form, grow or evade type II cell death. These conflicting roles make it difficult to determine how autophagy should be targeted therapeutically.[154]

At other times a different situation arises where the stimulation of autophagy by certain anti-cancer drugs leads instead to autophagy-based type II cell death (see Figure 7). This has been reported in various different cancer cell types. It is most clearly seen in cells where apoptosis has been inhibited, for instance in *bax-/-bak-/-* mouse embryonic

cells apoptosis inducers actually stimulate autophagic cell death, which is known to be autophagic as it is Beclin-1 and ATG-5 dependant. [155] This occurs as autophagy like other cellular processes must be tightly regulated, and while it can be beneficial to cells in a controlled way, if autophagy is up-regulated too much, the levels of catabolism surpass the levels of cellular anabolism, the result of which is cell death. Another possible reason for autophagic cell death under conditions of apoptotic inactivation/deficiency could be as a backup mechanism of cell death for the cell.

Autophagy has also been seen to have tumour suppressive effects (Figure 7), as it has been reported that loss of autophagy can lead to increased incidence of cancer. Various tumour suppressors such as p53, DAPK, PTEN and TSC1-TSC2 are known to induce autophagy, similarly some oncogenes like Ras and Akt suppress autophagy.[156] Certain types of cancer including breast, ovarian and prostate commonly have deletions of one allele coding for Beclin-1, a coiled-coil protein which interacts with Bcl-2, and stimulates autophagy by silencing the inhibitory actions of mTOR. The effects of this deletion have been studied in mice where it was seen to cause increased incidence of a range of cancers including lymphoma and lung cancer.[157] The reason behind this is thought to be due to the hypothesised protective effect of autophagy on genomic stability. It is thought that in cells lacking autophagic function, damaged organelles and misfolded proteins are allowed to build up. These damaged organelles, in particular peroxisomes and mitochondria become a source of reactive oxygen species that can lead to genotoxic stress. Essentially the cells become unable to buffer the metabolic stress and this can leave the cell susceptible to tumourigenesis. This theory is supported by work which reported that in mice allelic loss of Beclin-1 has been shown to increase the sporadic formation of tumours.[158] *In vitro* research has also shown that the loss of

Beclin in iBMK cells was associated with chromosomal losses and gains and gene amplification.[159]

Alternatively another mechanism to consider is that autophagy enables damaged cells to survive under conditions of metabolic stress, which may be another mechanism of promoting tumourigenesis by allowing these damaged cells to endure. [160] This has been examined *in vitro* where *beclin1*$^{+/-}$ cells were shown to be unusually large with abnormally shaped nuclei, when this was further examined it was seen that in cells containing only one beclin1 gene there was an increased incidence of chromosomal abnormalities including increased centromere number.[161]

Furthermore there is another proposed mechanism involving loss of autophagy and increased tumourigenesis. In cells where apoptosis is down-regulated, if autophagy is also restricted in some way and the cells are under conditions of metabolic stress (i.e. in the hypoxic area of a solid tumour), the cells can undergo necrosis. This necrosis can lead to inflammation due to the release of pro-inflammatory molecules like HMGB1 (High-mobility group protein B1) which attracts macrophages to the site. As the macrophages release chemokines and cytokines into the area this acts to increase proliferation and angiogenesis at the site (similar to wound healing) increasing the growth of the tumour.[162] Therefore in cancers where apoptosis is known to be down regulated the induction of autophagy could prevent wide spread necrosis and inflammation which would be conducive to tumour growth, and hence act to suppress the tumour.

The 'Autophagy Paradox' where both induction and inhibition of autophagy are found to have tumourigenic effects makes it difficult to conclusively say how autophagy should be targeted to treat cancer. However paradigms have been proposed to explain the

paradox. For instance, studies have shown that epithelial cancer cells can use oxidative stress to stimulate autophagy in the surrounding cancer associated fibroblasts. As oxidative stress induces autophagy this leads to the production of recycled nutrients within the fibroblasts. The fibroblast then excrete the recycled nutrients which can then be used by the tumour cells to stimulate growth even in the presence of low oxygen and other nutrient shortages.[163] This can be thought of as the tumour using the stroma as a sort of autophagic battery to provide energy to the tumour cells. If we are to accept this paradigm then it is clear how both the induction and inhibition of autophagy can both have anti-cancer effects. If autophagy was to be induced systemically then the stimulation of autophagy within the cancer cells would inhibit tumour growth, whilst the inhibition of autophagy systemically would disrupt the parasitic relationship by preventing the flow of recycled nutrients from the stromal cells to the cancer cells, again inhibiting growth. One of the benefits of this theory is that if autophagy inhibitors were being used to treat the cancer and resistance developed, treatment could switch to autophagy induction and vice versa. A combination treatment regime which alternated between the two could possibly prevent the development of drug resistance.[164] The reality is that autophagy may need to be treated differently depending on the type and stage of cancer, because if all theories are to be believed autophagy has multiple contradictory roles in both cancer growth and progression.

5.6 Autophagy and Apoptosis

To further complicate the matter it is now known that there is a certain amount of cross talk between autophagy and apoptosis, however, as before, this is not straight forward as the cross talk can involve both positive and negative feedback depending on the situation. In some cases autophagy can work with the apoptotic machinery to cause cell death. In cells where *beclin1* and *atg7* have been knocked down there is a reduction in

apoptosis seen.[165] However in other cases it appears that autophagy is only activated when apoptosis has been inhibited, an example of negative feedback between the two pathways. In this way autophagy acts as a 'back up' for when apoptosis fails. For example, this use of autophagy as a compensatory mechanism was seen under caspase-8 inhibition where stimulation of receptor-interacting protein and jun amino-terminal kinase, two molecules involved in sensing intra- and extracellular stress caused autophagic cell death.[166]

Another link between autophagy and apoptosis lies with p53. This 'genome guardian' is involved in both apoptosis and autophagy. p53 acts to stimulate apoptosis after sensing DNA damage, and therefore it is not surprising that nearly 50% of all cancers have some kind of mutation or deletion in the gene. Its role in autophagy is not so straightforward and p53 can be a positive regulator of autophagy when stimulated by genotoxic stress. For instance treatment of cells with a DNA damaging agent (etoposide) stimulates p53 which activates AMPK, which in turn inhibits mTOR leading to autophagy induction.[167] Furthermore under genomic stress p53 has also been reported to stimulate the transcription of DRAM (Damage-regulated modulator of autophagy) a highly conserved protein found on the lysosomal membrane which is essential for genomic stress-induced p53 activated autophagy and cell death. This effect was negated by knockdown of ATG5 thereby proving this effect to be dependent on the autophagic machinery.[168] However the relationship between p53 and autophagy is not always this simple. In other cases loss of p53 function either by pharmacological inhibition, knock out or knock down can in fact stimulate autophagy as well. This loss of p53 signalling leads to an increased rate of basal autophagy, interestingly this stimulation was seen to be almost maximal, meaning that autophagy could not be further enhanced by starvation.[169]

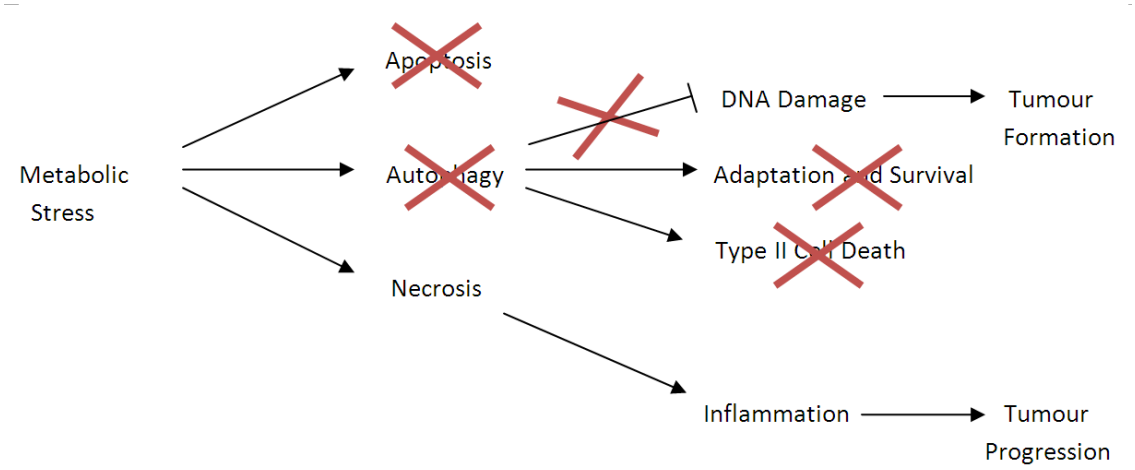


Figure 8. The consequences of autophagic inhibition in cancer. Many tumour types down regulate both autophagy and apoptosis. By inhibiting autophagy cells are unable to survive the low nutrient microenvironment often found in solid tumours, this would normally lead to apoptosis, however in some cancer cells which have down regulated the apoptotic pathway in order to prevent cell death, this can push the cells into undergoing necrosis which can then lead to further tumour progression. Furthermore by inhibiting autophagy this can also lead to an accumulation of damaged proteins and organelles within the cell resulting in creased oxidative stress and increasing the acquisition of mutations within the cell, some of which may be advantageous for cell growth and survival. [170]

While sometimes it seems that cancer cells undergo autophagy as a protective mechanism in order to survive nutrient shortages, other times certain anti-cancer drugs are able to promote cancer cell death via the autophagic pathways (autophagic cell death) (Figure 8). Therefore the challenge is to be able to characterise different cancers and the different stages of cancer to see where and when autophagy acts as a tumour suppressor and when it acts as a protective mechanism for cancer cells.

5.7 Measuring Autophagy

There are many methods that can be used to measure autophagy, each working to measure a different aspect of autophagy in a different model. Methods such as using the electron microscope to visualise the cell are useful for measuring the number of the autophagosomes present at a given time. If a measure of autophagic flux (the rate of

degradation of autophagic substrates) is needed then monitoring the steady state levels of autophagosomes, or punctate GFP-LC3, or LC3 levels using a western blot may not be appropriate. Instead there are several methods that directly monitor autophagic flux such as measuring the co-localisation of the autophagosome and the lysosome, the levels of autophagic substrates, and using chemical modulators of the autophagic process when carrying out methods such as western blots. The problem however with many of these methods is the interpretation of the results generated. Increases in levels of LC3-II generally mean an increase in autophagic activity however it can also be due to a reduced lysosomal degradation of the molecule.[171] Because of the complex nature of autophagy and the limitations of the above methods, it would seem prudent to use more than one method in order to look for corroboration between the two sets of results to avoid false positives and false negatives. All the methods have their disadvantages so it is best not to use them in isolation. The use of chemical modulators of autophagy and appropriate controls is key to understanding the results generated by many of the methods. By inhibiting a certain step in the autophagic process it is possible to validate the results, which enables conclusions to be made as to the exact cause of an increase or decrease in the target being monitored. For example, in protein degradation assays the degradation of long lived proteins may not be wholly due to autophagic degradation but also due to proteasomal degradation. Therefore by inhibiting autophagy it is possible to work out the extent to which it contributes and therefore work out the autophagic activity.[172]

When looking at spheroids, and in particular when looking at certain regions within a spheroid the most straight forward method for detecting autophagy would be immunohistochemistry. By staining for a molecule such as LC3-II or Beclin-1 it would be possible to examine and compare the expression within the different regions of the

spheroid. Additionally western blotting for LC3 or Beclin-1 would also be an invaluable tool for quantifying the expression and by splitting the spheroid into its respective layers (viable rim, necrotic core etc) using FACS, this would also make it possible to compare the expression at different locations within the spheroid. The electron microscopy is seen as the gold standard of autophagy detection, as the double membrane autophagosomes and autophagolysosomes can be visualised. As it is possible to confidently identify the autophagic vesicles, the results derived from using an electron microscope are more reliable than many of the other techniques. Other methods instead measure different markers of autophagy, markers which can be increased or decreased in response to other cellular processes other than autophagic flux, making the choice of appropriate controls crucial. The ability of electron microscopy to generate quantitative results is however somewhat reduced compared to other techniques, hence the need for multiple methods to provide a definitive conclusion.

6 Anoikis

Anoikis, a Greek word meaning "homelessness", is the process of apoptosis induced upon a cell's detachment from its surroundings. Cells rely upon their attachment to the extracellular matrix (ECM) and neighbouring cells not only for physical support but for signalling for a variety of processes, including proliferation, differentiation, gene expression, migration and continued survival. This exchange of information is made possible through integrins and transmembrane receptors which bind to the ECM and provide a scaffold for signalling molecules (Figure 9).

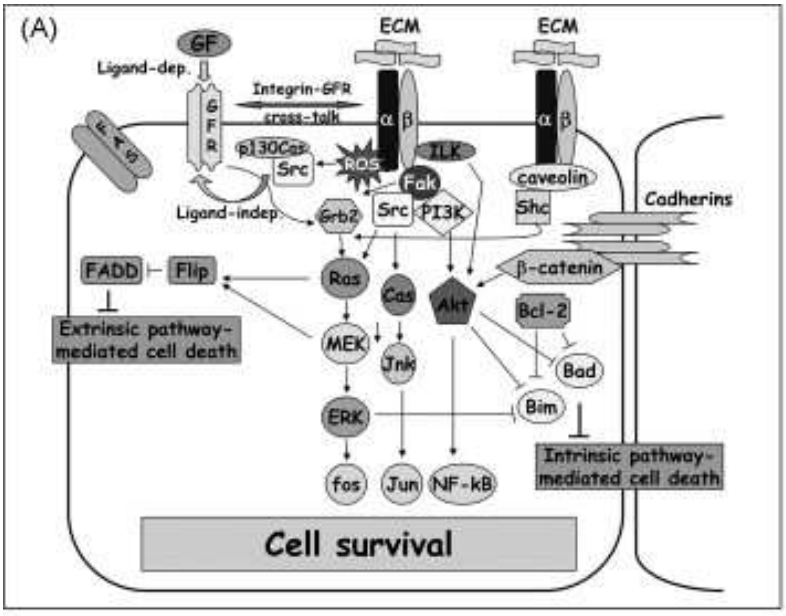


Figure 9. Diagrammatic representation of pro survival signalling mediated through cellular attachment. Integrin attachment to the ECM activates pro-survival signalling through a number of pathways shown. It also inhibits certain pro-apoptotic proteins. Cell-cell contacts involving Cadherins contribute to cell survival through the PI3K-Akt pathway causing a down regulation in BH3 proteins.

A cell's phenotype is highly dependent on the environment they exist in and is controlled by their interactions with the ECM.[173] When a cell loses contact with the ECM, its cell cycle is arrested and the cell then undergoes caspase-dependent cell death (apoptosis) (Figure 10). This is to ensure that any inappropriately placed cells are rapidly eradicated in order to prevent dyplasia within the tissue. [174] Anoikis is also used in tissue development, where it plays a role in the hollowing out of glands and other such involution process.[175]

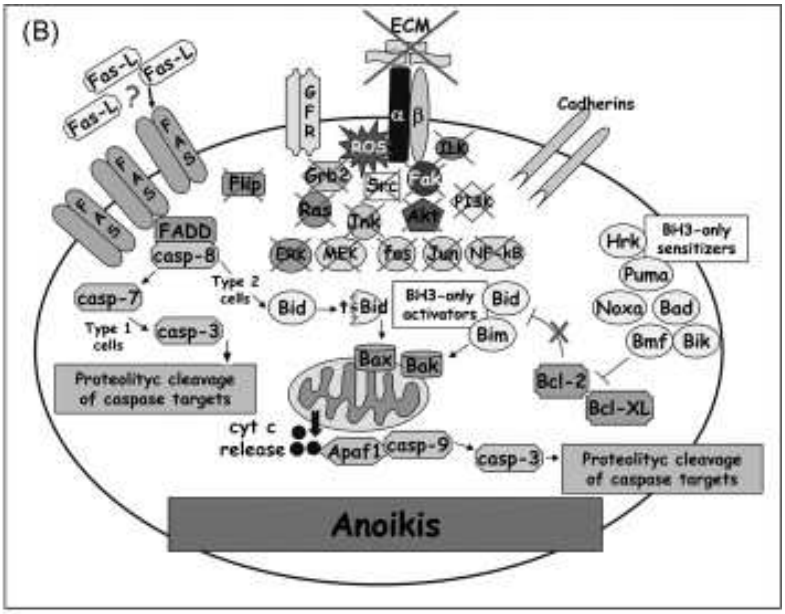


Figure 10. Diagrammatic representation of the mechanism underlying the cellular response to cellular detachment. Absence of ECM attachment fails to activate the pro-survival pathways. Lack of Cadherin mediated attachment to neighbours cells means a further lack of stimulation of the PI3K-Akt pathway. An increase in expression of Fas receptors on the cell surface further activates the extrinsic apoptotic pathway.

A cell's sensitivity to anoikis can be regulated. Not all cells react to a lack of anchorage by undergoing anoikis, fibroblasts for example do not. Tumour cells manage to resist anoikis through a number of different mechanisms (Figure 11). The resulting anchorage independent growth is necessary in order for cancer cells to metastasise as cells which have become anchorage independent then have the ability to travel throughout the body and form distant metastatic colonies.[176] Thus resistance to anoikis is emerging as a hallmark of metastatic cancer. Epithelial cells in particular are very sensitive to anoikis, though a reduction in this sensitivity has been documented in response to a number of different stimuli such as exposure to migratory factors, and transformation of the cell with certain oncogenes.[177] Resistance to anoikis has been found to promote malignancy in a number of different types of cancer including colorectal.[178, 179]

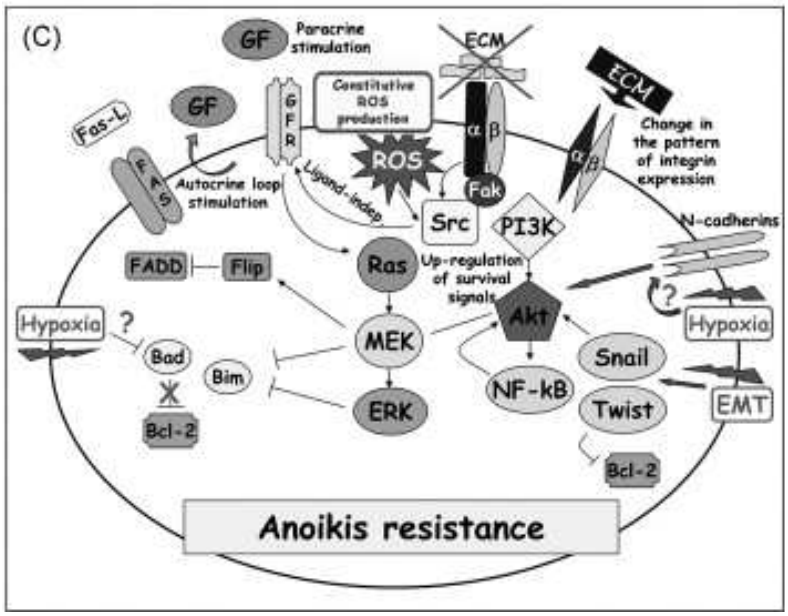


Figure 11. Summation of the various signalling molecules involved in anoikis resistance. Anoikis resistance is mediated through a number of different mechanisms. Survival pathways such as PI3K-Akt, MEK/ERK and NF-κβ can become constitutively activated through autocrine or paracrine signalling. Integrin expression can be modulated. Constitutive ROS production can also contribute to pro-survival signalling. EMT is also involved in anoikis resistance, up-regulation of several transcription factors involved in EMT, Snail and Twist, activate survival genes involved in the PICK-AKT signalling pathway. An induction in hypoxia may also survive to inhibit proapoptotic factors.

Several proteins found to have a role in cellular resistance to anoikis are involved in carcinogenesis.[180] Several of the proteins involved in the EMT are involved, including E-Cadherin, the down-regulation of which is known to circumvent anoikis through its relationship with the protein NRAGE.[181] Snail and Twist, two other proteins involved in the EMT, are also implicated in anoikis resistance both through their ability to regulate certain apoptosis control genes and to repress the expression of E-Cadherin.[182]

Another tactic cells employ to resist anoikis when metastasizing involves altering their expression of integrins. The expression of a differential complement of integrins can be induced through oncogenic signalling or in response to the local microenvironment the cell is currently residing in.[183, 184] By changing the expression of integrins to ones

appropriate for the new environment the cell can finally transduce ECM stimuli appropriately and hence suppress anoikis.[185] Conversely if a cell was not expressing the correct integrins for its current environment it could resist the induction of anoikis by 'ignoring' the signals which would usually lead to cell cycle arrest and cell death. By constitutively activating pro survival molecule such as PI3K,Rho-GTPase, Ras-Erk and NF-κβ the cells continue to survive and proliferate in the absence of integrin signalling.[186] To activate these pro survival factors the cells can employ autocrine secretion of a number of growth factors such as, HGF, bFGF, IL-8 and PDGF or over express certain receptors like EGFR and HGFR.[187, 188] Pro survival signalling can also be maintained in the absence of attachment by preventing the internalisation of signalling platforms located on the cell's surface. Under normal conditions these lipid rafts act to host the assembly and initiation of signalling pathways in response to the interaction between the ECM and the cell's integrins. When cells become unattached to the ECM the cell surface integrins are in an inactive state which causes the internalisation of the platforms and hence disrupts the pro survival signalling pathways. Caveolin-1 is required for the trafficking of the membrane platforms, loss of caveolin-1 leads to an inhibition of this process and hence prevents the disruption to cellular survival pathways. [189]

Autophagy has also been implicated in anoikis resistance in cancer cells. When the cell become detached from the ECM the reduction in integrin signalling stimulates autophagic signalling. Autophagy can then delay the onset of apoptosis in order to give the cell a chance to reach a new location and resume attachment to the ECM. Inhibition of autophagy results in reduced survival of detached cells and reduced viability in cells upon reattachment.[190, 191]

7 Senescence

Once a cell has reached the Hayflick Limit (maximum number of cell replications) it enters replicative senescence where the cell ceases cell division. Senescence was first described 5 decades ago when it was observed that normal cells have a limited replicative capacity in culture. A culture of cells was shown to gradually lose the ability to proliferate over time until ultimately all cells were growth arrested even in the presence of sufficient space, nutrients and growth factors. These non-dividing cells were however shown to remain viable for a substantial period of time following this arrest. In normal diploid cells the Hayflick Limit is determined by the shortening of the telomeres.[192] Other phenomenon can also induce cellular senescence such as toxins, oxidative stress, irradiation, DNA damage and certain oncogenes. This type of telomere independent senescence is termed premature senescence (PS). Senescent cells while they no longer replicate are still viable and do retain metabolic activity. The growth arrest seen in senescent cells is maintained in either the G1 or G2/M phase of the cell cycle with the help of increased expression of certain cyclin dependant kinase inhibitors (CDKIs).[193] The senescent phenotype includes a characteristic large, flattened cell morphology with a prominent nucleus and increased granularity of the cytoplasm (Figure 12). Alterations in the cell's gene expression profile and changes in the cell's secretion profile are also seen, termed the senescence-associated secretary phenotype.[194]

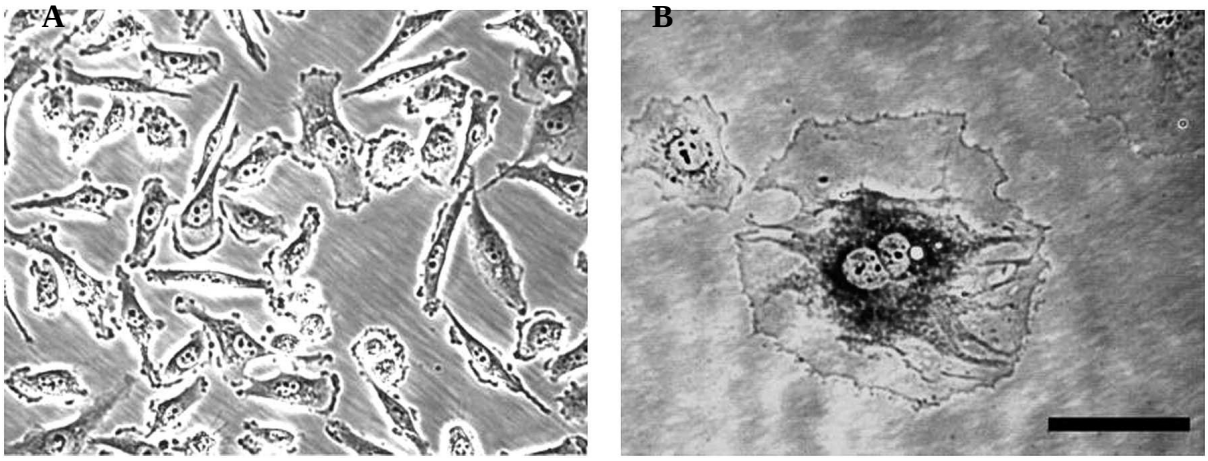


Figure 12. Senescent cell morphology. Panel A represents a normal proliferating PC3 cell. Panel B shows the flattened and round morphology of a senescent PC3 cell. Scale bar represents 100μm.[195]

Cellular senescence has now been recognised as a potent tumour suppressor mechanism similar to that of apoptosis. [196] As senescence prevents cells from proliferating indefinitely, cancer cells must be capable of evading the process in order to proliferate. Benign melanocytic nevi are one example of where senescence prevents the expansion of the cell population. The moles initially form due to increased activity of the mutant oncogene BRAF (v-raf murine sarcoma viral oncogene homolog B1) which causes an increase in proliferation, the melanocytes then undergo growth arrest accompanied by the $p16^{Ink4a}$ expression and stain positive for senescence markers.[197] Senescence has also been detected in lung adenomas but is absent in adenocarcinomas, indicating that senescence could function as a mechanism for preventing malignant transformation.[198] Most cancer cells are immortal, the majority of which evade senescence through a mechanism involving the re-activation of the telomerase genes.[199] Lack of senescence associated tumour suppressors such as p53 and retinoblastoma protein is not however enough to suppress senescence under certain conditions such as ionizing radiation or exposure to chemotherapeutic drugs.[200, 201] Chemotherapy induced senescence is termed Therapy Induced Senescence (TIS) and has been recognized in a subset of cells in many

different tumour types after chemotherapy or radiation treatment.[202] TIS may represent a back up mechanism for growth arrest in tumours which are apoptosis resistant. In an *in vivo* setting induction of TIS has been linked to a better post treatment prognosis. [203]

7.1 Detecting Senescence

Senescence can be induced in cells through a variety of different stimuli, but regardless of the stimulus, senescence can be detected *in vitro* and *in vivo* using several methods. The 'gold standard' for detecting senescence in cells is the SA-β-Gal assay which involves staining for the presence of senescence associated β-galactosidase activity. This assay requires active lysosomal activity and hence necessitates the use of fresh or frozen tissue making it incompatible with IHC detection methods in archived paraffin embedded samples. Other detection methods can be used when the SA-β-Gal assay is inappropriate, these tend to rely on the characteristic changes in a cell when it becomes senescent. A long term exit from the cell cycle is also a hallmark of cellular senescence along with changes in the cell's morphology. Morphological changes are more easily seen in cells *in vitro* as opposed to in tissues.[204]

To detect senescence in tissue samples particularly those that have been paraffin embedded, several senescence-associated markers can be used. In fibroblasts, senescence-associated heterochromatic foci (SAHF) areas of highly condensed chromatin can be stained for. Under senescence, SAHF form and can be detected using DNA binding dyes such as DAPI and Haematoxylin.[205] Furthermore certain chromatin associated proteins localise to these SAHF and can be stained for to detect the regions.[206] Markers of DNA damage can also be used to identify senescent cells specifically double strand breaks. DNA damage can result in the presence of DNA damage foci which have been found to be characterised by an ATM/ATR activation which leads to the phosphorylation of Ser139 on histone H2AX molecules which

localises to the site of damage.[207, 208] Staining procedures for the expression of phosphorylated H2AX have been used to detect senescence. In conjugation with negative Ki-67 staining, γ-H2AX staining has been shown to detect senescent cells *in vitro* in fibroblasts and *in vivo* in hepatocytes[209] and intestinal sections.[210]

7.2 How cancer might use senescence to survive

The role of senescence in cancer is a complicated one but recently it has been suggested that senescence might not just represent a tumour suppressive mechanism but may also have tumour promoting effects. Premature senescence is considered a DNA damage response for cancer cells subjected to chemotherapy.[211] While exit from the cell cycle and growth arrest has a cytostatic effect on tumour cells, evidence has shown that senescent tumour cells are not as benign as originally thought. Senescent cells can promote the growth of their neighbouring cancer cells by the cytokines they secrete.[212, 213] Moreover senescent cells by their very nature are resistant to many forms of cytotoxic chemotherapeutic drug which target actively proliferating cells. Most importantly cancer cells have been shown to be capable of re-entering the cell cycle following acquisition of a senescent phenotype.[214] These cells' escape mechanism has been found to involve various proteins. Survivin has been linked to the process through its relationship with the Cdc2/Cdk1 complex, the complex is responsible for phosphorylating Survivin enabling its senescence escaping capabilities. Cells which escape senescence are known to over express Cdc2/Cdk1, and this expression directly effects their viability.[215] Twist a protein involved in the EMT has also been revealed to be involved. It was found to be able to override senescence through the abrogation of the inhibition of the cell cycle via p21 and p16. The effects of this are twofold; the cell escapes senescence and also undergoes EMT producing a viable, proliferative and invasive cancer cell.[216] The survival of senescent cancer cells and their return to a

proliferative cell state is clearly a cancer promoting mechanism and could affect the efficacy of anti-cancer treatments, thereby making it a possible target for the future.

7.3 Autophagy and Senescence

Studies have shown a link between autophagy and senescence. As both autophagy and senescence can be induced by the same stimuli, a link between the two processes was not unexpected. Autophagy has been found to contribute to the cellular state of senescence and up regulation of autophagy and autophagy related genes has been seen to occur during senescence. Furthermore inhibition of autophagy using autophagy inhibitors has been found to have an inhibitory effect upon the induction of a senescent state.[217] It has been suggested however than autophagy can switch between its cell survival function and its cell death function (autophagic cell death) by switching between senescence and apoptosis. [218]

8 Tumour Dormancy

The vast majority of cancer deaths are the result of a metastatic form of the disease.[219] Metastatic deposits form from disseminated tumour cells which often remain dormant for a substantial period of time. The term 'dormancy' was first used to describe the prolonged latent phase in the development of disease progression from primary tumour to secondary metastatic deposits in the mid 20th century by pathologist Rupert A. Willis.[220] Tumour dormancy is quite common, for instance in breast and prostate cancer 20-45% of patients will relapse years or decades later.[221, 222] In fact many different cancer types are associated with persisting disseminated cells which can result in residual disease post treatment.[223] How these disseminated cells remain dormant and why is not fully understood though there has been research into the area in recent years.

Tumour dormancy can be divided into two main categories. First, tumour mass dormancy whereby the expansion of the tumour cells as a population is inhibited for example by restrictions in the blood supply. Second, tumour cell dormancy where the tumour cells are in a state of growth arrest or quiescence.[224] In the following section tumour cell dormancy will be discussed.

8.1 Dormancy: Senescence or Quiescence?

The mechanisms underpinning tumour cell dormancy have not been fully elucidated, yet those which are often attributed to the phenomenon include quiescence and senescence. Senescence which tends to function as a tumour suppressive mechanism has been detected through positive S-β-Gal staining in patient tumour samples following chemotherapy.[225] However for a tumour to perpetuate it needs to have acquired the ability to bypass senescence, though it is possible that some parts of the senescent machinery can still be induced in the tumour cells. Whether or not it is the senescent cells detected in tumours which are responsible for the tumours relapse is unknown. Conversely quiescence might be a better suited[226]. Quiescence has been seen in cancer stem cells and in cells which have entered G_0 growth arrest due to insufficient growth stimulatory signals, and more so than senescence it is easily reversible.[227]

8.2 Tumour Cell Dormancy Manifestations

It is a commonly held view that some cancer cells within a primary tumour will have metastatic capabilities, and that these traits are acquired over the period of time that it took the tumour mass to develop from a mass of proliferating cells (hyperplasia) into an established tumour.[228] However more recent research has suggested that dissemination of tumour cells may occur far earlier than previously thought, preceding the acquisition of a full complement of metastatic abilities. [229] These disseminated cells then progress through the acquisition of the metastatic phenotype whilst separate from the tumour

mass. If these disseminated cells, which managed to survive chemotherapeutic intervention, were to continue to proliferate continuously then the time before relapse would be much shorter than often observed. The best explanation for why this does not occur is tumour cell dormancy. The explanations for the lag time in return to cellular proliferation could be due to a number of different possible reasons. The niche in which the cancer cell has lodged itself could be conducive for a quiescent state, or the stress of a new microenvironment could induce growth arrest. The cell might then be able to remodel the new niche in order to produce an environment more conducive to growth.[230, 231] Another possible explanation for the lag time in the development of metastases is the initial lack of sufficient vasculature. Prior to the development of a tumour vasculature the cells within a metastatic deposit can only proliferate so much before outgrowing the current vasculature, after this areas of necrosis can form as the rate of growth/proliferation balances the rate of cell death. This is termed angiogenic dormancy.[232]

8.3 Clinical Implications of Tumour Dormancy

Disseminated tumour cells are hard to detect owing to their low numbers. Whilst they are commonly found in the circulation and bone marrow they can also lodge themselves in other organs not routinely checked. Furthermore due to their non-proliferative state, dormant cancer cells are intrinsically resistant to many common chemotherapeutics which target actively dividing cells. For example studies carried out *in vivo* found that disseminated breast cancer cells which were tagged with GFP (green fluorescent protein) were both growth arrested and resistant to the commonly used chemotherapeutic Doxorubicin.[233] In order to better detect and treat dormant disseminated cells better biomarkers need to be found that will help detect the low abundance cells throughout the body. Treatments which are either effective on non

proliferative cells or cause the cells to exit their dormant phase and become active once more, are needed.

In order for an epithelial tumour cell to disseminate it must first acquire mesenchymal properties. Furthermore when activated a once dormant epithelial tumour cell will only be successful in forming a metastatic deposit if it has undergone the Epithelial Mesenchymal Transition (EMT). Both the EMT and its role in cancer will be discussed in the next section.

9 Epithelial Mesenchymal Transition

The EMT is a morphogenetic process that was first discovered following studies on the development of complex structures in embryonic development.[234] The beginnings of a metazoan organism starts with epithelial cells organised in a single layer. Polarized epithelial cells form continuous sheets that are interconnected by specific structures, tight junctions, gap junctions, desmosomes, hemidesmosomes and adherens junctions. The basal-apical polarity is perpendicular to the plane of the epithelium and influenced by the cells' association with the basement membrane, allowing the exchange of nutrient and waste products between external and internal compartments. At some point in the early stages of embryonic development mesenchymal cells develop from the epithelium. Mesenchymal cells in contrast to epithelial cell display front-rear polarity (see Figure 13) and rarely have any point of physical contact with other mesenchymal cells. [235] Unlike epithelial cells mesenchymal cells have the ability to migrate and invade through both epithelial cells and other mesenchymal cells, with the front-rear polarity enabling their locomotive capabilities. The conversion of an epithelial cell into a mesenchymal cell is termed the epithelial mesenchymal transition and was first defined in the 1980s.[236]

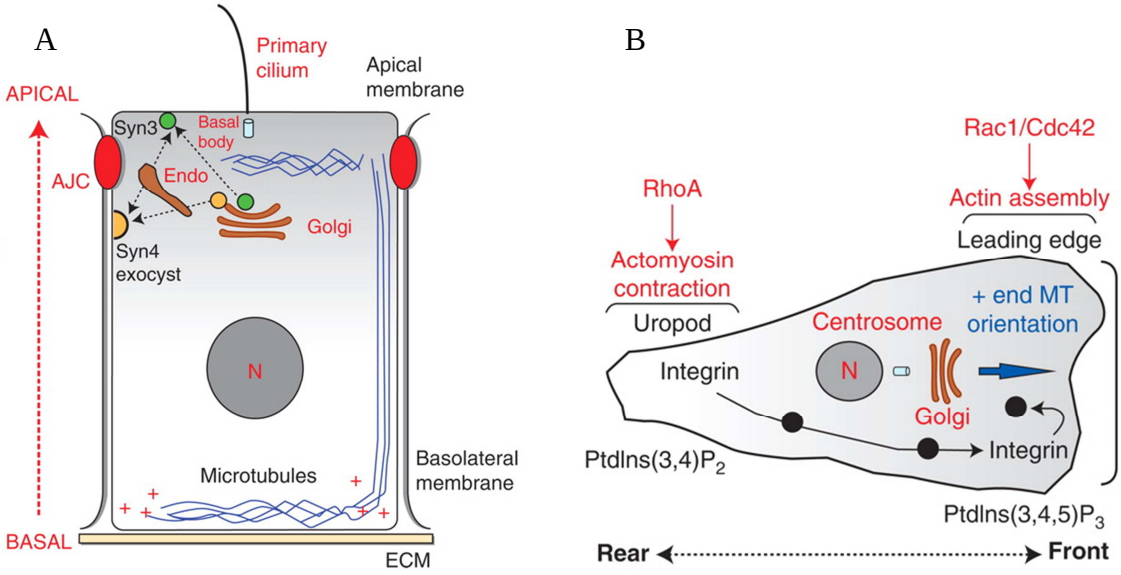


Figure 13. Cellular Polarity and Epithelial Mesenchymal Cell Phenotype. Panel A Apical-basal Polarity, polarized epithelial cells have an distinct apical basal polarity in an orientation perpendicular to their attachment to the basement membrane (ECM). Organelles and various internal structures are organised in response to this polarity. Panel B Front-rear polarity, mesenchymal cells are organised based upon polarity between the front end (leading edge) and rear (uropod) of the cell. Certain organelles are orientated to the front of the cell, including centrosome, microtubules and golgi body, while the endocytic and exocytic pathways are orientated to the rear.[237]

EMT is required for many different processes, through embryonic development into normal adult physiological process, including mesoderm formation, neural crest and heart valve development during embryogenesis and the formation of fibroblasts during adulthood.[238] Passage through the EMT is not irreversible, once the cells has gone through the change there is the process of Mesenchymal-Epithelial Transition (MET) which reverses the transformation. This just serves to highlight the plasticity of cells in terms of their phenotype and functional capabilities.[239]

The process of EMT requires three profound changes to the phenotype of the epithelial cells. They must first un-attach themselves from their neighbouring epithelial cells by severing the various connections that anchor them to their neighbours. In order to do

this they must change their expression of differentiation markers, from the epithelial type cell-cell adhesion junction proteins and cytokeratin intermediate filaments to mesenchymal type fibronectin, vimentin filaments and certain integrins,[240] and different spicing variants of certain cell surface receptors like FGFR2.[241] Loss of the molecule E-Cadherin and other components from tight junctions, is sufficient to reduce the attachment and change the polarity of the cell.[242] The cell must then undergo a change in morphology from the typical polygonal cobblestone shape to the mesenchymal spindle shape. Lastly the cell must undergo certain changes in it's functional characteristics in order to enable it to invade through ECM.[243] While not all changes are present in all cases of EMT, the one functional hallmark of EMT is the ability to migrate through the ECM.

9.1 The role of the EMT in Cancer

The detection of EMT in cancer cells is usually carried out by measuring the expression of proteins known to be involved in the process. Loss of E-Cadherin and gain of N-Cadherin is characteristic of cells having gone through the EMT, along with expression of other molecules including vimentin, snail, slug, twist, various integrins and matrix metalloproteinases (MMPs). The transcriptional regulator Snail is central to the repression of E-Cadherin and its protein levels are also measured to determine EMT. Loss of E-Cadherin has been widely documented in epithelial tumours, furthermore the blocking of E-Cadherin has been shown in *in vitro* cell systems to be enough to trigger EMT in epithelial cell types.[244] The loss of E-Cadherin in epithelial tumours has also been shown to trigger the swift conversion from a benign adenoma to an invasive carcinoma. Inherited mutations in the E-Cadherin gene are known to predispose to gastric cancer, loss of expression of the protein in sporadic tumours is linked with poor prognosis. [245] As Snail acts to repress E-Cadherin it is understandable that the

expression of the two proteins is inversely correlated at the invasive edge of tumours.[246] Snail along with Twist, another transcriptional regulator, also have roles in promoting expression of mesenchymal markers such as N-Cadherin.[247]

10 Cancer Stem Cells

Stem cells are defined as undifferentiated cells with limitless replicative potential which have the ability to give rise to differentiated daughter cells. Cancer stem cells are therefore defined are tumourigenic (capable of forming a tumour) and have been proven to be capable of forming a completely heterogeneous tumour.[248] Types of tumour cells which are known to have undergone the EMT include cancer stem cells. Cancer stem cells are strongly implicated in metastasis as they are able to disseminate and survive the journey to a new target tissue and then have the ability to form a new heterogeneous tumour mass.[249, 250] The links between EMT and stem cells in the context of cancer will be discussed further in subsequent sections.

10.1 Stochastic vs. Hierarchical Growth

Originally it was believed that tumours grew through a stochastic model where all cells within the tumour had equal tumour forming abilities. The theory hypothesises that tumours arise due to the accumulation of mutations, specifically activation mutations in oncogenes and inactivation mutations within tumours suppressor genes. In colorectal cancer this model is called the Vogelstein model. The model states that it is the normal epithelial cells lining the gut that are the origin of colorectal cancer after undergoing a sequential series of genetic mutations which change their proliferation and self renewal capabilities.[251]

More recently evidence has been found that suggests that the stochastic theory is less likely and instead a hierarchical theory based on cancer stem cells has been proposed.

The cancer stem cell hypothesis is a contentious issue, it hypothesises that there is a population of quiescent cells which upon depletion of the majority of the tumour mass, via chemotherapy, are able to repopulate the tumour. It is only these so called cancer stem cells which are believed to have the ability to initiate and maintain a tumour and then metastasise.[252] These tumour initiating cells are believed to be stem cells or stem-like cells due to their ability to differentiate into phenotypically heterogeneous progeny. Differentiated daughter cells with a limited replicative lifetime are produced from undifferentiated highly clonogenic parent stem cells which have a limitless replicative potential. Therefore indicating that growth and propagation of the tumour is determined by a small sub-population of cells. This hierarchical model is similar to the growth of normal tissues, for instance colon crypts are known to undergo continuous regeneration driven by the population of crypt intestinal stem cells (ISC).

This model suggests that a tumour is made of two main populations of tumour cells, those that have through differentiation lost their ability to propagate a new tumour and those that remain undifferentiated and retain this clonogenic ability.[253] Furthermore pathways known to be involved in self renewal of non cancerous stem cells such as Wnt signalling in colon derived stem cells, are also found to be involved in colorectal cancer suggesting that tumours are indeed derived from the stem cell compartment.[254]

10.2 Evidence for Cancer Stem Cells in Solid Tumours

Until recently there was little known about cancer stem cells in solid tumours. While haematopoietic stem cells found in the blood were relatively easy to access, accessing stem cells within solid tumours is more difficult. The need for markers to identify them is crucial. Breast cancer was the first solid tumour from which cancer stem cells were identified. Using $CD44^{high}/CD24^{low}$ as markers, cells were isolated which were then capable of initiating a tumour *in vivo* with implantation numbers as low as 200. The

cells were found to be capable of regenerating the heterogeneity of the original tumour. This demonstrated that the cells had a stem cell's capacity for self renewal, proliferation and differentiation.[255]

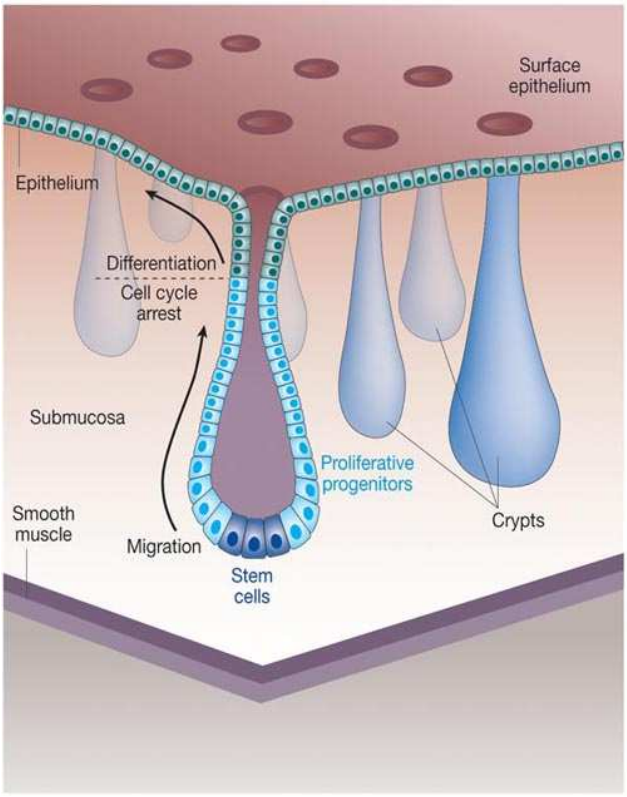


Figure 14. Structure of the Cypts of Lieberkühn in the colon. Showing the location of stem cells at the base of the crypt and the cycle of proliferation and differentiation upwards through the crypt.[256]

Cancer Stem cells have also been found in colorectal cancer, identified using a downstream target of Wnt, Lgr5.[257] Using lineage tracing it was discovered that these Lgr5 expressing cells were capable of differentiating into all cell types found in the intestinal crypt.[258]

It is known that normal stem cells exist in the intestinal crypts. The colon is organised into 4 layers, the outermost epithelial layer is one cell thick and organised into finger like projections called the crypts of Lieberkühn. The function of the crypts is to increase surface area. This entire layer is replenished once every 5 days.[259] The upper parts of the crypt are composed of terminally differentiated cells which gradually migrate up the crypt until they are shed into the intestinal lumen. These differentiated cells are derived from multi-potent stem cells which reside at the base of the crypt (Figure 14). The stem cells divide asymmetrically to produce two daughter cells, one remains undifferentiated to retain the stem cell phenotype and the other differentiates into one of the three types of epithelial cell found in the crypt. The three epithelial lineages found in the crypt are

the goblet cell which is involved in producing mucus, enterendocrine cells and absorptive enterocytes. This cycle of cellular proliferation and differentiation is tightly controlled through the Wnt signalling pathway.[260]

10.3 The colorectal cancer stem cell model

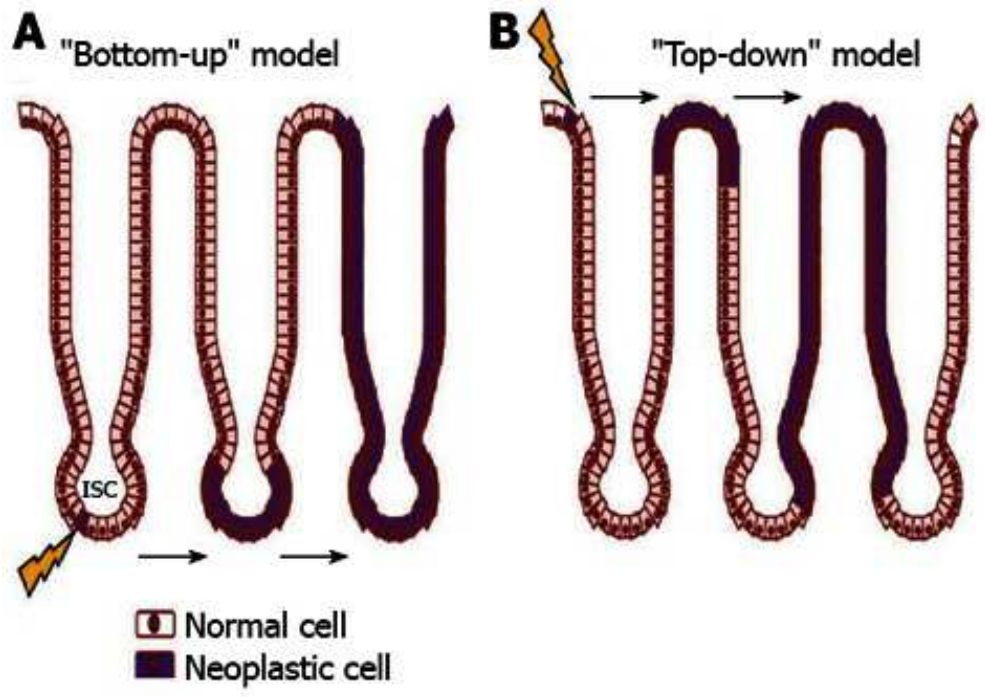


Figure 15. Proposed models for the colorectal cancer stem cell. Panel A Bottom-up model where the cell of origin is an intestinal stem cell located in the base of the crypt. Panel B Top-down model where the cell of origin is located at the top of the crypt.[261]

There are two different hypotheses for the mechanism involving stem cells by which it is believed colorectal cancer develops (Figure 14). The 'Top-down' model hypothesises that the cell of origin is a progenitor or differentiated cell found in the upper levels of the crypt. It is this cell which acquires a number of mutations which give it a cancer stem cell-like phenotype. Histological evidence for this using small colorectal adenomas showed that while cells at the top of the crypt had APC (adenomatous polyposis coli) mutations, these same genetic alterations were not seen in the intestinal stem cells.

Furthermore these differentiated cells were not found to be clonally related to the stem cells.[262]

The 'Bottom-up' model suggests that the origin of tumourigenesis is the intestinal stem cell found at the bottom of the crypt. The stem cell is the first to acquire gain of function mutations which lead to anomalous proliferation producing more cancer stem cells and other progeny, which lead to further cancer cells devoid of any ability to self renew. Evidence supporting this theory includes studies which have shown that deletion of both APC alleles specifically in intestinal stem cells using BMi1-, CD133- and Lgr5-Cre recombinase mice leads to the formation of tumours. Yet when the same deletions were performed in differentiated cells tumours did not form.[263]

Figure 16. Model of sporadic adenoma polyp formation. APC is inactivated in a single cell (see arrow) at the base of the crypt (Top). The transformed cell then begins to proliferate and passively migrates upwards to the top of the crypt as part of the routine process of epithelial cell turnover (Middle). When the transformed cells reach the top of the crypt they continue to spread into the normal epithelium and surrounding crypts pushing the normal cells down and gradually replacing them in the crypts in a top-down mechanism (Bottom).[264]

While neither hypothesis has been disproved there are theories which explain how both may in fact occur. Evidence has shown that transformed cells are present at the top of the crypt in small adenomas while the bottom of the crypt remains unchanged yet it has been suggested that the cell origin may still be the intestinal stem cell. Once transformed in the base of the crypt this malignant stem cell could have migrated to the top and then spread throughout the intestinal epithelial layer

to surrounding crypts where it then invaded the crypts in a top-down movement (Figure 16).

10.4 Cancer Stem Cell Properties

Cancer stem cells are believed to be defined by three particular properties; the ability to self renew indefinitely, the ability to independently give rise to a heterogeneously diverse tumour, and the expression of a number of stem cell markers. Cancer stem cells are known to be extremely tumourigenic with the ability to form serially transplantable tumours from primary tumours in immunocompromised mice.[265] While cancer stem cells are known to be able to form a tumour *in vivo* most of the cells making up the bulk of the tumour are not.[266] One of the most clinically important characteristics of cancer stem cells is their resistance to chemotherapeutics, a characteristic which makes them critically important in the role of tumour recurrence. Stem cells have several key features which are involved in their chemoresistance. They are not highly proliferative, in fact they tend to exist in a quiescent state until needed, as shown by their retention of BrdU labelling.[267, 268] As many anti cancer drugs are targeted against proliferating cancer cells this makes them inherently resistant to them. Also stem cells are known to differentially express a number of signalling molecules which are involved in chemoresistance. Cancer stem cells are also thought to evade chemotherapeutic induced cell death by the expression of multiple drug efflux pumps in their cell membranes such as Multidrug Resistance Transporter 1 (MDR1) and Adenosine triphosphate-binding cassette ($ABCB_1$).[269] An increased DNA damage repair response has been discovered to be the cause for increased resistance in the response to radiotherapy. Cancer Stem Cells are able to activate DNA damage checkpoints more easily than normal cells and in fact have been found to have a higher basal level of activation of the DNA damage

checkpoint.[270, 271] The Wnt/β-Catenin pathway is also believed to be involved in cancer stem cells increased radioresistance.[272]

Colon cancer stem cells are known to express Bcl-2 at higher levels and this results in an increased resistance to apoptosis.[273] Increased expression of IL-4 has also been shown to provide protection against drug induced apoptosis in colon cancer stem cells via its modulation of cell death pathways. In particular it has been shown to increase resistance of cancer stem cells against 5-Fluorouracil, a resistance which can be diminished with the use of IL-4 blocking antibodies.[274, 275] Aldehyde dehydrogenase (ALDH1) is also known to be up regulated in cancer stem cells, so much so that it is a marker for cancer stem cells.[276] ALDH is an enzyme which oxidises aldehydes and therefore protects cells against alkylating agents. The increased expression seen in cancer stem cells has been shown to confer resistance to anti cancer drugs such as cyclophosphamide.[277, 278] Altered metabolism within CSCs is also thought to be involved in their increased survival.[279]

This chemoresistance is partly why cancer stem cells are implicated in the recurrence of tumours after chemotherapeutic intervention. Once the majority of the tumour has been eradicated using an anti-cancer drug the chemoresistant cancer stem cells are enriched within the population as they managed to survive and go on to repopulate the tumour. This expansion of the stem cell pool within the tumour would make it more likely that the resulting secondary tumour would be chemoresistant as it is made up from daughter cells of chemotherapy resistant cancer stem cells.[280] Therefore to stop tumour re-growth and metastasis it is necessary to ensure that all cells within a tumour including the quiescent cancer stem cells have been targeted and killed. In order to do this a targeted therapy must be developed which takes advantage of the differences within cancer stem cells to selectively kill them. One of the ways to do this is by looking for

cancer stem cell markers as a means of targeting new or existing drugs specifically to the cancer stem cells.

10.5 Stem Cell Markers

As mentioned previously ALDH is known to be a marker of cancer stem cells, in particular ALDH1 is known to identify colorectal cancer stem cells.[281] Other identified colorectal cancer stem markers include epithelial cell adhesion molecule EpCAM, CD44 and CD166. Research has shown colorectal cancer cells expressing these three markers to be capable of engrafting *in vivo* in immunocompromised mice to form heterogeneously differentiated tumour similar to the parent tumour. [282] CD44 is known to be not just a stem cell marker but is actively involved in the tumourigenic process, CD44 is a glycoprotein involved in cell adhesion, proliferation and differentiation.[283] In hepatocellular carcinoma targeting CD44 has been shown to induce apoptosis specifically in the cancer stem cell population.[284] Genetic knockout of CD44 in mice prone to intestinal tumours shows a reduction in tumour occurrence.[285] Hence CD44 is an example of a stem cell marker which might be useful not only for identifying cancer stem cells but also as a way of selectively killing them.

CD133 is also a stem cell marker in a number of different tumour types. In colorectal cancer CD133 has been shown to be important for identifying cells with tumour initiating capabilities. CD133 positive cells were shown to be able to establish tumours *in vivo*, as well as maintain an undifferentiated population while allowing some CD133 cells to differentiate to establish tumour heterogeneity. On the other hand CD133 negative cells were found to be unable to initiate tumour growth *in vivo*.[286] CD24 is another cell surface antigen found to be expressed on colorectal cancer stem cells, it has been found to be correlated with increased invasiveness[287] drug resistance[288] and the ability to form heterogeneous tumours *in vivo*.[289] Even when working with defined

stably expressed stem cell markers it is difficult to identify cancer stem cells due to their low number within tumours. This means that very large numbers of tumour cells must be assayed in order to find cancer stem cells.

10.6 Stem cells and the microenvironment: the stem cell niche

Stem cell niches were first discovered when it became clear that stem cells exist in specific stable microenvironments.[290] Normal stem cells reside in this stem cell niche where their undifferentiated state is maintained with help from the microenvironment.[291] Stem cells niches are groups of cells residing in a special location that functions to maintain a population of stem cells. A niche in particular acts to anchor the stem cells in a specific location using various adhesion molecules to tether the stem cells to the extracellular matrix (see Figure 17). The niche also has a role in maintaining a certain number of stem cells by the use of extracellular factors to control their proliferation and lineage fate. Numerous different signalling molecules have been implicated in this process including Wnt, Notch, FGF and BMP.[292]

While normally a niche would restrain stem cell division by supplying anti proliferative signalling, if a mutation were to arise which made the stem cells resistant to anti proliferative signalling or self sufficient in growth signals this could then lead to uncontrolled cell proliferation and possibly tumourigenesis.

The stem cell niche has also been implicated in the process of tumourigenesis. This is due the niche's role in maintaining stem cell populations. The niche anchors the stem cells in the specific microenvironment, this anchorage is composed of cadherin and β-catenin adhesion molecules which form complexes to hold the cells in place.[293] There is a large amount of commonality between the mechanisms and signalling molecules involved in regulating this anchorage process and those involved in cancer cell invasion

and metastases. For instance matrix metalloproteinases such as MMP-9 are involved in activation and translocation of stem cells in the bone marrow.[294] MMPs are also known to be involved in the invasion and metastases of cancer cells through their roles in extracellular matrix degradation.[295] Integrin signalling is also involved in the migration of stem cells in both haematopoietic and neural stem cell niches,[296, 297] as well as being involved in the metastasis of cancer cells.[298]

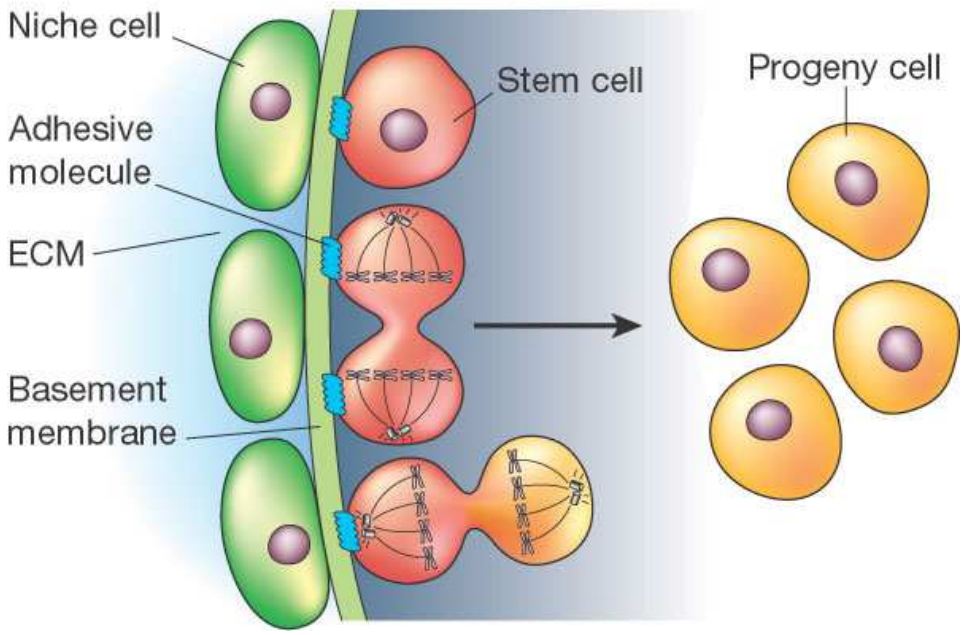


Figure 17. The Stem Cell Niche, depicts stem cell anchored to the basement membrane via adhesion molecules which relay signals to the stem cells controlling their division and blocking differentiation. As seen at the bottom when a lineage specific signal is received the stem cell divides so that one daughter cell maintains its connection with the basement and hence maintains its undifferentiated status. Above is shown a stem cell undergoing cell division to produce two new stem cells. [299]

10.7 Stem Cells: EMT and Metastasis

The majority of patients do not die from their primary tumour but as a consequence of metastasis.[300] Cancer stem cells are also known as cancer initiating cells due to their ability to initiate a primary tumour, also due to their abilities to repopulate a heterogeneous tumour after chemotherapeutic assault. They are also able to avoid

anoikis when detached from surrounding cells, therefore it is straightforward to extrapolate that cancer stem cells are the cells which first form metastatic deposits. In order for this to be possible the cancer stem cells must be able to detach from their surroundings, migrate and invade into blood vessels, survive within the circulation and then disseminate into new tissues to form a secondary tumour. This cellular behaviour is indicative of a cell undergoing the epithelial mesenchymal transition, a process undergone by cells before they are capable of metastasising.[301] Mesenchymal cells have a scaffolding/anchoring function and have multiple roles in wound healing and embryonic development and unlike epithelial cells are resistant to anoikis.[302]

Evidence has suggested that metastatic cancer cells that have undergone EMT may express a cancer stem-like phenotype. Pleural effusions were found to contain disseminated breast cancer cells which expressed a breast cancer stem cell phenotype ($CD44^{high}/CD24^{low}$).[303] In fact CD44 happens to be a β-catenin/TCF target gene further supporting the link between EMT and cancer stem cell phenotype. Induction of EMT in immortalised mammary epithelial cells caused expression of a number of stem cell markers as seen previously, and in addition showed increased mammosphere formation, an ability consistent with breast cancer stem cells. This phenomenon was also investigated from the opposite angle, stem-like cells which were isolated from mammary carcinomas were investigated for EMT markers which were found to be expressed.[304] Further evidence has been seen in pancreatic cancer where CD133 expressing cells with a mesenchymal phenotype were found on the invasive edge of tumours, these cells were also found to express the chemokine receptor CXCR4 known to be involved in the metastatic spread of cancer.[305]

This link between EMT and stem cell properties help to explain how the process of metastasis is possible in light of the cancer stem cell hypothesis. It is understood that

before a cell can metastasise it must first undergo the EMT to give it the required migratory phenotype in order to detach from its surrounding, avoid anoikis and travel to a distant site. However what isn't clear is how it is able to complete the last step of metastasis, formation and growth of the new tumour, especially since most metastases manage to recapitulate the structure and organisation of the primary tumour.[306] While the EMT gives the cell the required capabilities to migrate to the new site, self renewal which the majority of the cells making up the bulk of the tumour lack makes it difficult to understand how a metastatic deposit would be formed. However the discovery that the EMT induces many of the properties of stem cells including self renewal, provides an answer. Patients which were found to have stem cell markers such as ALDH1 on circulating tumour cells in the blood have a worse prognosis than those who do not.[307]

10.8 The Migrating Cancer Stem Cell Model

All this evidence lead to the Migrating Cancer Stem Cell Model. It is proposed that there are two different types of stem cell, the stationary cancer stem cell and the migratory cancer stem cell. Whilst the stationary cancer stem cells is thought to be located within the mass of tumour embedded in epithelial tissue, the migratory cancer stem cell is believed to be located on the periphery of the tumour on the boundary between tumour and host tissue. The migratory cancer stem cells are derived from their counterparts the stationary cancer stem cells, but have acquired EMT-like characteristics. In colorectal cancer the migratory stem cells have been characterised as having high levels of nuclear β-catenin (consistent with having undergone the EMT). Levels of these cells at the tumour-host boundary have been found to be correlated with metastasis and poor survival.[308,309] These cells then have all the necessary traits for metastasis; the EMT enables them to disseminate from the original tumour and survive

the journey to a new metastatic site, and the stem cell functionality allows them to form metastatic deposits at the new site (Figure 18).

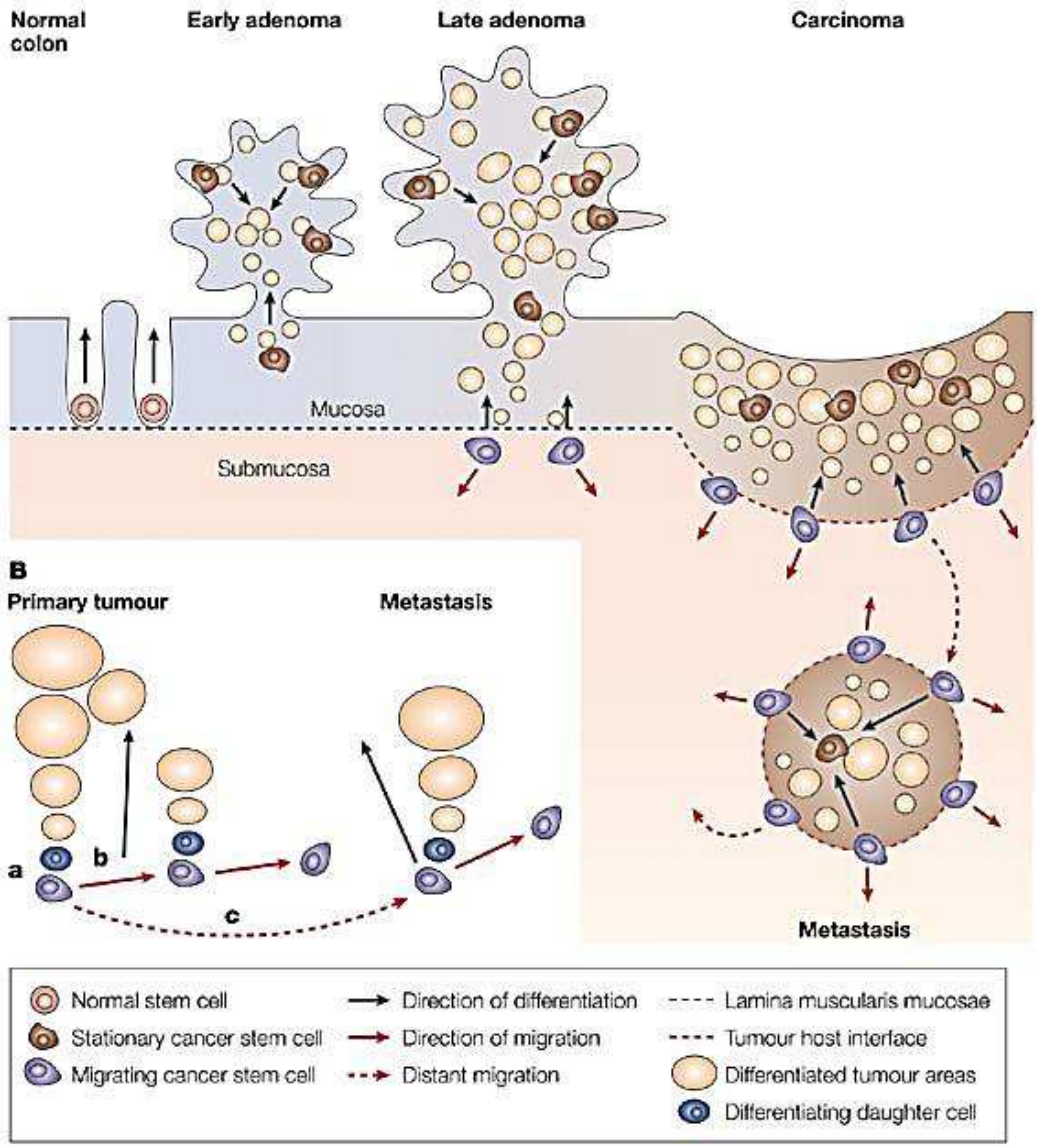


Figure 18. The Migrating Cancer Stem Cell Model. Panel A Normal intestinal stem cells are located at the base of the crypts in normal colon. Stationary stem cells are found in benign adenomas though remain detectable in more differentiated tumours and metastatic deposits. The step from benign to malignant occurs as the EMT is induced in tumour cells specifically the stationary stem cells to produce migrating cancer stem cells. Panel B Migrating cancer stem cells divide asymmetrically to produce to daughter cells, one of which is set on a path of differentiation and proliferation (a), the other either migrated a small distance before beginning another asymmetric division (b) thereby contributing the mass of the original tumour or migrates a greater distance to begin the formation of a metastatic deposit (c).[310]

10.9 Stem Cells and Autophagy

Stem cells have be found to be more reliant on autophagy under conditions of low oxygen and nutrient availability than their counterpart normal cancer cells. In pancreatic cancer, *in vitro* starved cancer stem cells were shown to have increased survival under hypoxic conditions compared to non stem cell starved cancer cells. Furthermore after survival under hypoxic conditions the cancer stem cells went on to change morphology to a fibroblast phenotype and this was accompanied by an increase in their migratory potential as measured by the wound healing assay. This behaviour is consistent with the cells having undergone the EMT stimulated by the harsh microenvironment. It was found that in response to hypoxia and starvation the cancer cells up-regulated their levels of autophagy, this process was found to be critical for their survival under these conditions. When autophagy was inhibited the cancer stem cells underwent apoptosis. Inhibition of autophagy has also been shown to inhibit the self renewal capabilities of stem cells as shown by reduced sphere formation *in vitro* and tumour formation *in vivo,* suggesting that the hypoxic tumour microenvironment is an inducer of an increased migratory cancer stem cell.[311] Similar evidence has also been found in liver cancer stem cells where higher levels of autophagy in response to hypoxia and nutrient deprivation were seen in CD133 positive cells compared to CD133 negative cells. And as with the pancreatic cells the liver cancer stem cells were shown to have increased survival capabilities in the hostile environment compared to the normal cancer cells, a survival ability which was also impaired by the inhibition of autophagy.[312, 313]

Autophagy also has a role in the maintenance of quiescence and senescence within stem cells, by removing damaged macromolecules which would otherwise cause a loss of quiescence within the cell and suppressing mitochondrial biogenesis and ROS production.[314]

Autophagy has also been implicated in the survival of colorectal cancer stem cells. Stem cells isolated using cell surface markers CD44 and CD24 were found to have higher colony forming abilities, these same cells showed an increased resistance when exposed to paclitaxel. This resistance was related to the autophagic flux within the cell. Resistant cells were shown to have a higher level of autophagy and when autophagy was inhibited the cytotoxicity of paclitaxel was increased.[315] The discovery that autophagy may be involved in cancer stem cells increased survival ability in a toxic tumour microenvironment may lead to new ways in which to selectively target them.

10.10 Stem Cell Assays

The use of stem cell markers for identifying cancer stem cells is not straightforward. As with normal stem cells in different organs, cancer stem cells from different tumour types express different combinations of markers. The stem cell markers which characterise a glioma cancer stem cell would not be the same as those which characterise a colorectal cancer stem cell, though there is some cross over. To further complicate the issue, cancer stem cells do not stably express the same markers all the time and marker expression can be varied depending on the microenvironment in which the stem cell is currently growing. To make the use of markers more robust a combination of different marker expression is often used, along with experimental evidence from various functional *in vivo* and *in vitro* assays which measure the stem-like characteristics displayed by the cells. [316]

The gold standard for stem cell assays is the serial transplantation assay, which is used to show the self renewal and multi-lineage differentiation capacity of the cells (Figure 19). The assay involves transplanting cells into orthotopic sites of immunocompromised mice to determine xenograft formation. To assay self renewal cells from the xenograft are then transplanted into a second animal and so on with the resulting tumours sharing

similar histological phenotypes. [317] While this assay remains the best option for determining stem cell like behaviour of cells it is both time consuming and expensive. In vitro assays therefore have been developed to try and measure the same stem cell characteristics in a more rapid and quantitative way.

In 1996 a significant breakthrough in stem cell research resulted in the discovery that undifferentiated multipotent neural cells could be grown and maintained in suspension without loss of proliferation of multilineage potential. This discovery led to the introduction of the neurosphere assay.[318] Since then this sphere formation assay has been used to measure the 'stemness' of stem cells in many different tissue[319, 320, 321] and cancer types.[322, 323, 324] The sphere formation assay, which measures the ability of single cells to form spheroid like structures in non adherent conditions, is dependent on the stem cells ability to avoid anoikis. Sphere formation assays are conducted under conditions for stem cells growth which normally involved serum free media and growth factors such as Epidermal Growth Factor. [325]

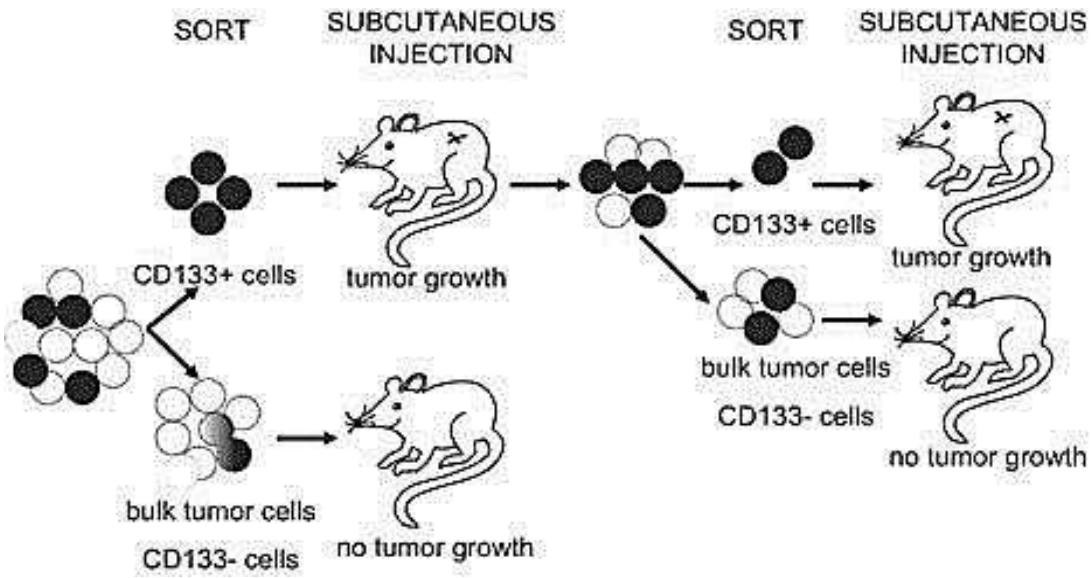


Figure 19. Diagram depicting the serial transplantation assay used to identify cancer stem cells. Using a marker such as CD133 the ability of these cells to initiate and repopulate a heterogeneous tumour can be measured.[326]

10.11 How to target cancer stem cells

The discovery and characterisation of cancer stem cells has major implications for the treatment of cancer. Treatment of colorectal cancer is difficult and various regimens exist including surgery, radiotherapy, cytotoxic drugs and monoclonal antibodies against VEGF and EGFR. However in advanced cases of the disease most treatments are not curative. The reason behind this is that most conventional therapies are targeted against actively proliferating or cycling cancer cells which compose the bulk of the tumour, but which excluded the cancer stem cells. Cancer stem cells are also known to be more resistant to chemotherapeutics through other mechanisms including increased drug efflux pumps and increased DNA repair. Treatments which decrease the proportion of normal cancer cells whilst sparing the cancer stem cell population only serve to enrich the stem cell population in tumours.[327] Therefore in order to successfully treat colorectal cancer a method must be developed to target the cancer stem cells.

There are various different ways to target cancer stem cells. One method is to force them to terminally differentiate thereby resulting in their loss of tumourigenic potential.[328] The effect of differentiation therapy would be to cause the cancer stem cells to develop into mature cancer cells which no longer have the ability to initiate further tumour growth. Compounds such as salinomycin have been found to induce terminal epithelial differentiation in breast cancer stem cells thereby reducing the percentage of stem cells within the breast cancer population; this has been shown to inhibit tumour growth *in vivo*.[329]

The use of stem cell markers to target the cancer stem cells is not as straight forward as it seems. While the markers are specific to cancer stem cells and therefore absent in normal cancer cells they are often found to be expressed by a wide variety of cells in normal non-cancerous tissue. This would mean that any drug targeted against that

marker would have a very small therapeutic window as it would also target the healthy tissues, an effect which would limit its use. However success has been seen *in vivo* for the targeting of CD44.[330] The choice of which stem cell marker to use through its distribution in healthy tissues and the cancer stem cells reliance upon it is crucial.

Elucidating some of the mechanisms for multidrug resistance employed by cancer stem cells has lead to further ways to selectively target the cells. Cancer stem cells are known to over express certain drug effluxers such as the ABC transporters. Inhibition of the these transporters in combination with a classical chemotherapeutic agent would selectively target the stem cells. This method has shown to be successful in melanoma.[331] Other drug resistance mechanisms such as the expression of IL-4 have also been exploited; in colorectal cancer pre-treatment of CD133 positive cells with an IL-4 neutralising antibody was shown to increase apoptosis in response to chemotherapeutics both *in vitro* and *in vivo*.[332]

Another mechanism which could be used to target cancer stem cells is their ability for self renewal. By targeting the evolutionarily conserved pathways known to be involved in this process, such as Wnt and Notch signalling it is possible to induce cell death in these cancer stem cells.[333] However these signalling pathways are also relied on by normal stem cells so there is an issue with selectivity.

11 Synopsis

In summation clinical data has established a link between tumour necrosis and poor prognosis, though the mechanisms surrounding the relationship are unknown. Recent evidence has found active enzyme activity, along with mRNA synthesis, to be occurring in the necrotic core of MCTS; therefore warranting further investigation.[334]

The topics discussed in this chapter include a number of different physiological processes that are implicated in cancer progression and metastasis that could help to explain the link between tumour necrosis and poor prognosis. Whilst the biological and therapeutic implications of the hypoxic tumour microenvironment have been deduced the necrotic microenvironment is still waiting to be investigated.

How the cells manage to survive within the poor conditions found in necrotic tissue is an important question which when answered could highlight new therapeutic targets. The various different processes by which a cell could use to survive such a microenvironment need to be investigated. Such processes include the EMT which could be used to survive low attachment conditions; resistance to anoikis can be mediated through this process, and entering the cellular state of senescence or conversely becoming dormant for a time to survive the low oxygen and nutrient conditions. Metabolic adaptations such as the induction of autophagy can be used by the cell to survive the low nutrient conditions, the properties of cancer stem cells including both increased survival capabilities in response to poor conditions and the induction of EMT could also help explain the survival of cells with necrotic tissue.

By investigating each of these cellular mechanisms and their role in the tumour microenvironment specifically in necrotic cells it may be possible to more fully elucidate their role. This thesis therefore will work towards proving or disproving the hypothesis that viable cells within areas of necrosis are more aggressive and treatment resistant. And that their existence explains why tumour necrosis is associated with poor prognosis and overall survival in cancer patients, hence suggesting that necrotic tissue must also be considered when treating cancer.

12 Aims

It is currently understood that necrosis, which is characteristic of large solid tumours, involves the disruption of the cell membrane and release of cellular contents into the extracellular space. The result of which can be promotion of further cancer growth, invasion and progression.[335] Necrosis has been shown to lead to the acquisition of a more aggressive phenotype and chemoresistance in a range of different tumour types, including breast and gastrointestinal.[336, 337] Due to recent evidence that cells in areas of necrosis are actively expressing mRNA and proteins,[338] the focus of this research is to understand how and why tumour necrosis is linked to poor progression and aggression in tumours. The primary objective of this research is to investigate the necrotic core of the MCTS. Particularly to try and determine whether or not it is simply a mass of dead cells or whether it harbours different populations of cells which have adapted to live in the harsh environment. These adaptations could possibly make the cells in some way more resilient and possibly aggressive than the cells found on the viable rim. By establishing whether there are any biological differences in these cells it may be possible to determine whether this has any therapeutic significance and may lead to a new biological target. Furthermore it would go towards explaining why high levels of necrosis have been linked to increased aggression in some solid tumours.

13 References

[1] Worldwide Cancer Key Facts. (2014). Retrieved May, 2012, from http://publications.cancerresearchuk.org/publicationformat/formatfactsheet/worldwidekeyfacts.html

[2] Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100(1):57-70.

[3] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5): 646-74.

[4] Faloon WW, Gorham LW. Clinical experience with nitrogen mustard. *N Y State J Med.* 1948;48(6):612-6.

[5] Gilman A. The initial clinical trial of nitrogen mustard. *Am J Surg*. 1963;105:574-8.

[6] DeVita Jr DT, Chu E. A History of Cancer Chemotherapy. *Cancer Res.* 2008;68:8643-8653.

[7] Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer.* 2013;13(10):714-26.

[8] Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature*. 1973 Jun 1;243(5405):290-3.

[9]Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, Capdeville R, Talpaz M. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N Engl J Med*. 2001;344(14):1038-42.

[10] Gerber DE. Targeted therapies: a new generation of cancer treatments. *Am Fam Physician*. 2008;77(3):311-9.

[11] Floor SL, Dumont JE, Raspe E. Hallmarks of cancer: of all cancer cells, all the time? *Trends Mol Med*. 2012;18(9):509–515.

[12] Sermeus A, Michiels C. Reciprocal influence of the p53 and the hypoxic pathways. *Cell Death Dis.* 2011;2:e164.

[13] Proskuryakov SY, Konoplyannikov AG, Gabai VL. Necrosis: a specific form of programmed cell death?. *Exp. Cell Res*. 2003;283 (1): 1–16.

[14] Robbins and Cotran: Pathologic Basis of Disease, 8th Ed. 2010. Pg. 14-23.

[15] Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: a molecular definition. *Oncogene*. 2004;23(16):2825-37.

[16] Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nuñez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G; Nomenclature Committee on Cell Death 2009. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. *Cell Death Differ*. 2009;16(1):3-11.

[17] Overholtzer M, Mailleux AA, Mouneimne G, Normand G, Schnitt SJ, King RW, Cibas ES, Brugge JS.A nonapoptotic cell death process, entosis, that occurs by cell-in-cell invasion. *Cell*. 2007;131(5):966-79.

[18] Yu SW, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, Dawson VL. Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science*. 2002;297(5579):259-63.

[19] Andrabi SA, Kim NS, Yu SW, Wang H, Koh DW, Sasaki M, Klaus JA, Otsuka T, Zhang Z, Koehler RC, Hurn PD, Poirier GG, Dawson VL, Dawson TM. Poly(ADP-ribose) (PAR) polymer is a death signal. *Proc Natl Acad Sci U S A*. 2006;103(48):18308-13.

[20] Stevens A., Lowe J, Young B, and Health J. (2003). Wheater's Basic Histopathology: A Colour Atlas and Text. 4th ed. london: Churchill Livingstone.

[21] Huether S E, McCance K L. 5th Ed. 2011. Understanding Pathophysiology. Missouri: Mosby Elsevier.

[22] Robbins and Cotran: Pathologic Basis of Disease, 8th Ed. 2010. Pg. 14-23.

[23] Robbins and Cotran: Pathologic Basis of Disease, 8th Ed. 2010. Pg. 14-23.

[24] Caruso R, Parisi A, Bonanno A, Paparo D, Quattrocchi E, Branca G, Scardigno M, Fedele F. Histologic coagulative tumour necrosis as a prognostic indicator of aggressiveness in renal, lung, thyroid and colorectal carcinomas: A brief review. *Oncol Lett*. 2012;3(1):16-18.

[25] Robbins and Cotran: Pathologic Basis of Disease, 8th Ed. 2010. Pg. 14-23.

[26] Cho YS, Park SY, Shin HS, Chan FK. Physiological consequences of programmed necrosis, an alternative form of cell demise. *Mol Cells*. 2010;29(4):327-32.

[27] Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol*. 2000;1(6):489-95.

[28] Proskuryakov SY, Konoplyannikov AG, Gabai VL. Necrosis: a specific form of programmed cell death? Exp Cell Res. 2003;283(1):1-16.

[29] Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W, Vandenabeele P. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *J Exp Med*. 1998;187(9):1477-85.

[30] Schulze-Osthoff K, Krammer PH, Dröge W. Divergent signalling via APO-1/Fas and the TNF receptor, two homologous molecules involved in physiological cell death. *EMBO J*. 1994;13(19):4587-96.

[31] Gabai VL, Meriin AB, Yaglom JA, Wei JY, Mosser DD, Sherman MY. Suppression of stress kinase JNK is involved in HSP72-mediated protection of myogenic cells from transient energy deprivation. HSP72 alleviates the stewss-induced inhibition of JNK dephosphorylation. *J Biol Chem*. 2000;275(48):38088-94.

[32] Mochizuki T, Asai A, Saito N, Tanaka S, Katagiri H, Asano T, Nakane M, Tamura A, Kuchino Y, Kitanaka C, Kirino T. Akt protein kinase inhibits non-apoptotic programmed cell death induced by ceramide. *J Biol Chem*. 2002;277(4):2790-7.

[33] Shimizu S, Eguchi Y, Kamiike W, Waguri S, Uchiyama Y, Matsuda H, Tsujimoto Y. Retardation of chemical hypoxia-induced necrotic cell death by Bcl-2 and ICE inhibitors: possible involvement of common mediators in apoptotic and necrotic signal transductions. *Oncogene*. 1996;12(10):2045-50.

[34] Tan J, Town T, Placzek A, Kundtz A, Yu H, Mullan M. Bcl-X(L) inhibits apoptosis and necrosis produced by Alzheimer's beta-amyloid1-40 peptide in PC12 cells. *Neurosci Lett*. 1999;272(1):5-8.

[35] Leist M, Single B, Castoldi AF, Kühnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med*. 1997;185(8):1481-6.

[36] Lopez-Meraz ML, Niquet J, Wasterlain CG. Distinct caspase pathways mediate necrosis and apoptosis in subpopulations of hippocampal neurons after status epilepticus. Epilepsia. 2010;51 Suppl 3:56-60.

[37] Leist M, Single B, Castoldi AF, Kühnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med*. 1997;185(8):1481-6.

[38] Faraco PR, Ledgerwood EC, Vandenabeele P, Prins JB, Bradley JR. Tumor necrosis factor induces distinct patterns of caspase activation in WEHI-164 cells associated with apoptosis or necrosis depending on cell cycle stage. *Biochem Biophys Res Commun*. 1999;261(2):385-92.

[39] Ruemmele FM, Dionne S, Levy E, Seidman EG. TNFalpha-induced IEC-6 cell apoptosis requires activation of ICE caspases whereas complete inhibition of the caspase cascade leads to necrotic cell death. *Biochem Biophys Res Commun*. 1999;260(1):159-66.

[40] Mayhew TM, Myklebust R, Whybrow A, Jenkins R. Epithelial integrity, cell death and cell loss in mammalian small intestine. *Histol Histopathol*. 1999;14(1):257-67.

[41] Barkla DH, Gibson PR. The fate of epithelial cells in the human large intestine. *Pathology*. 1999;31(3):230-8.

[42] Murdoch WJ, Wilken C, Young DA. Sequence of apoptosis and inflammatory necrosis within the formative ovulatory site of sheep follicles. *J Reprod Fertil*. 1999;117(2):325-9.

[43] Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol*. 2000;1(6):489-95.

[44] Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, Mehlen P, Nuñez G, Peter ME, Piacentini M, Rubinsztein DC, Shi Y, Simon HU, Vandenabeele P, White E, Yuan J, Zhivotovsky B, Melino G, Kroemer G. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death. *Cell Death Differ*. 2012;19(1):107-20.

[45] Smith CC, Davidson SM, Lim SY, Simpkin JC, Hothersall JS, Yellon DM. Necrostatin: a potentially novel cardioprotective agent? *Cardiovasc Drugs Ther*. 2007;21(4):227-33.

[46] Welz PS, Wullaert A, Vlantis K, Kondylis V, Fernández-Majada V, Ermolaeva M, Kirsch P, Sterner-Kock A, van Loo G, Pasparakis M. FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation. *Nature.* 2011;477(7364):330-4.

[47] Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB. Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes Dev*. 2004;18(11):1272-82.

[48] Bano D, Young KW, Guerin CJ, Lefeuvre R, Rothwell NJ, Naldini L, Rizzuto R, Carafoli E, Nicotera P. Cleavage of the plasma membrane Na+/Ca2+ exchanger in excitotoxicity. *Cell*. 2005;120(2):275-85.

[49] Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G, Hedrick SM, Gerber SA, Lugovskoy A, Yuan J. Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat Chem Biol*. 2008;4(5):313-21.

[50] Christofferson DE, Yuan J. Necroptosis as an alternative form of programmed cell death. *Curr Opin Cell Biol*. 2010;22(2):263-8.

[51] Lin Y, Choksi S, Shen HM, Yang QF, Hur GM, Kim YS, Tran JH, Nedospasov SA, Liu ZG. Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. *J Biol Chem*. 2004;279(11):10822-8.

[52] Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science*. 2004;304(5676):1500-2.

[53] Bell BD, Leverrier S, Weist BM, Newton RH, Arechiga AF, Luhrs KA, Morrissette NS, Walsh CM. FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells. *Proc Natl Acad Sci U S A.* 2008;105(43):16677-82.

[54] Pollheimer MJ, Kornprat P, Lindtner RA, Harbaum L, Schlemmer A, Rehak P, Langner C. Tumor necrosis is a new promising prognostic factor in colorectal cancer. *Hum Pathol*. 2010;41(12):1749-57.

[55] Fisher ER, Anderson S, Redmond C, Fisher B. Pathologic findings from the National Surgical Adjuvant Breast Project protocol B-06. 10-year pathologic and clinical prognostic discriminants. *Cancer*. 1993;71(8):2507-14.

[56] Fisher CJ, Egan MK, Smith P, Wicks K, Millis RR, Fentiman IS. Histopathology of breast cancer in relation to age. *Br J Cancer*. 1997;75(4):593-6.

[57] Pollheimer MJ, Kornprat P, Lindtner RA, Harbaum L, Schlemmer A, Rehak P, Langner C. Tumor necrosis is a new promising prognostic factor in colorectal cancer. *Hum Pathol*. 2010;41(12):1749-57.

[58]Gao JF, Arbman G, Wadhra TI, Zhang H, Sun XF. Relationships of tumor inflammatory infiltration and necrosis with microsatellite instability in colorectal cancers. *World J Gastroenterol*. 2005;11(14):2179-83.

[59] Hiraoka N, Ino Y, Sekine S, Tsuda H, Shimada K, Kosuge T, Zavada J, Yoshida M, Yamada K, Koyama T, Kanai Y. Tumour necrosis is a postoperative prognostic marker for pancreatic cancer patients with a high interobserver reproducibility in histological evaluation. *Br J Cancer*. 2010;103(7):1057-65.

[60] Lam JS, Shvarts O, Said JW, Pantuck AJ, Seligson DB, Aldridge ME, Bui MH, Liu X, Horvath S, Figlin RA, Belldegrun AS. Clinicopathologic and molecular correlations of necrosis in the primary tumor of patients with renal cell carcinoma. *Cancer*. 2005;103(12):2517-25.

[61] Langner C, Hutterer G, Chromecki T, Leibl S, Rehak P, Zigeuner R. Tumor necrosis as prognostic indicator in transitional cell carcinoma of the upper urinary tract. *J Urol*. 2006;176(3):910-3.

[62] Muro-Cacho CA, Cantor AB, Morgan M. Prognostic factors in malignant gastrointestinal stromal tumors. Ann Clin Lab Sci. 2000;30(3):239-47.

[63] Llombart-Bosch A, Contesso G, Henry-Amar M, Lacombe MJ, Oberlin O, Dubousset J, Rouëssé J, Sarrazin D. Histopathological predictive factors in Ewing's sarcoma of bone and clinicopathological correlations. A retrospective study of 261 cases. *Virchows Arch A Pathol Anat Histopathol*. 1986;409(5):627-40.

[64] Swinson DE, Jones JL, Richardson D, Cox G, Edwards JG, O'Byrne KJ. Tumour necrosis is an independent prognostic marker in non-small cell lung cancer: correlation with biological variables. *Lung Cancer*. 2002;37(3):235-40.

[65] Kessler R1, Gasser B, Massard G, Roeslin N, Meyer P, Wihlm JM, Morand G. Blood vessel invasion is a major prognostic factor in resected non-small cell lung cancer. *Ann Thorac Surg*. 1996;62(5):1489-93.

[66] Swinson DE, Jones JL, Richardson D, Cox G, Edwards JG, O'Byrne KJ. Tumour necrosis is an independent prognostic marker in non-small cell lung cancer: correlation with biological variables. *Lung Cancer*. 2002;37(3):235-40.

[67] Leek RD, Landers RJ, Harris AL, Lewis CE. Necrosis correlates with high vascular density and focal macrophage infiltration in invasive carcinoma of the breast. *Br J Cancer*. 1999;79(5-6):991-5.

[68] Leek RD, Landers RJ, Harris AL, Lewis CE. Necrosis correlates with high vascular density and focal macrophage infiltration in invasive carcinoma of the breast. *Br J Cancer*. 1999;79(5-6):991-5.

[69] Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell*. 2012;21(3):309-22.

[70] Raza A, Franklin MJ, Dudek AZ. Pericytes and vessel maturation during tumor angiogenesis and metastasis. *Am J Hematol.* 2010;85(8):593-8.

[71] Pietras K, Ostman A. Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res*. 2010;316(8):1324-31.

[72] Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell*. 2010;141(1):39-51.

[73] Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer*. 2008;8(8):618-31.

[74] Pavlides S, Vera I, Gandara R, Sneddon S, Pestell RG, Mercier I, Martinez-Outschoorn UE, Whitaker-Menezes D, Howell A, Sotgia F, Lisanti MP. Warburg meets autophagy: cancer-associated fibroblasts accelerate tumor growth and metastasis via oxidative stress, mitophagy, and aerobic glycolysis. *Antioxid Redox Signal*. 2012;16(11):1264-84.

[75] Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res.* 1989;49(23): 6449-65.

[76] Warren, BA (1979). The vascular morphology of tumors. H.I Petersen (Ed.), Tumor blood circulation, Boca Raton, FL: CRC Press Inc. 1-48.

[77] Brown JM, Wilson WR. Exploiting tumour hypoxia in cancer treatment. *Nat Rev Cancer.* 2004;4(6):437-47.

[78] Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature*. 1996 Jan 4;379(6560):88-91.

[79] Erler JT, Cawthorne CJ, Williams KJ, Koritzinsky M, Wouters BG, Wilson C, Miller C, Demonacos C, Stratford IJ, Dive C. Hypoxia-mediated down-regulation of Bid and Bax in tumors occurs via hypoxia-inducible factor 1-dependent and -independent mechanisms and contributes to drug resistance. Mol Cell Biol. 2004 Apr;24(7):2875-89.

[80] Rouschop KM, van den Beucken T, Dubois L, Niessen H, Bussink J, Savelkouls K, Keulers T, Mujcic H, Landuyt W, Voncken JW, Lambin P, van der Kogel AJ, Koritzinsky M, Wouters BG. The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. *J Clin Invest.* 2010;120(1):127-41.

[81] Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nat Rev Cancer*. 2011;11(2):85-95.

[82] Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med*. 2003;9(6):677-84.

[83] Hill RP, Marie-Egyptienne DT, Hedley DW. Cancer stem cells, hypoxia and metastasis. *Semin Radiat Oncol.* 2009;19(2):106-11.

[84] Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer Cell*. 2003;3(4):347-61.

[85] Teicher BA. Hypoxia and drug resistance. *Cancer Metastasis Rev*. 1994;13(2):139-68.

[86] Carroll VA, Ashcroft M. Targeting the molecular basis for tumour hypoxia. *Expert Rev Mol Med*. 2005;7(6):1-16.

[87] Peters KB, Brown JM. Tirapazamine: a hypoxia-activated topoisomerase II poison. *Cancer Res*. 2002;62(18):5248-53.

[88] Patterson LH, McKeown SR. AQ4N: a new approach to hypoxia-activated cancer chemotherapy. *Br J Cancer.* 2000;83(12):1589-93.

[89] Wilson WR, Hay MP. Targeting hypoxia in cancer therapy. *Nat Rev Cancer.* 2011;11(6):393-410.

[90] Boyle RG, Travers S. Hypoxia: targeting the tumour. *Anticancer Agents Med Chem*. 2006;6(4):281-6.

[91] Höckel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst*. 2001;93(4):266-76.

[92] Vaupel P. Hypoxia and aggressive tumor phenotype: implications for therapy and prognosis. *Oncologist*. 2008;13 Suppl 3:21-6.

[93] Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer*. 2002;2(8):563-72.

[94] Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer*. 2002;2(8):563-72.

[95] Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verástegui E, Zlotnik A. Involvement of chemokine receptors in breast cancer metastasis. *Nature*. 2001;410(6824):50-6.

[96] Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, Porter D, Hu M, Chin L, Richardson A, Schnitt S, Sellers WR, Polyak K. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell*. 2004;6(1):17-32.

[97] Oh P, Li Y, Yu J, Durr E, Krasinska KM, Carver LA, Testa JE, Schnitzer JE. Subtractive proteomic mapping of the endothelial surface in lung and solid tumours for tissue-specific therapy. *Nature*. 2004;429(6992):629-35.

[98] Mueller-Klieser W. Three-dimensional cell cultures: from molecular mechanisms to clinical applications. *Am J Physiol*. 1997;273:C1109–23.

[99] Yuhas JM, Tarleton AE, Harman JG. In vitro analysis of the response of multicellular tumour spheroids exposed to chemotherapeutic agents in vitro or in vivo. *Cancer Res.* 1978;38:3595–8.

[100] Mueller-Klieser WF, Sutherland RM. Oxygen tensions in multicell spheroids of two cell lines. *Br J Cancer*. 1982;45(2):256-64.

[101] Bloch K, Smith H, van Hamel Parsons V, Gavaghan D, Kelly C, Fletcher A, Maini P, Callaghan R. Metabolic Alterations During the Growth of Tumour Spheroids. *Cell Biochem Biophys*. 2013 Sep 14.

[102] Brat DJ, Castellano-Sanchez AA, Hunter SB, Pecot M, Cohen C, Hammond EH, Devi SN, Kaur B, Van Meir EG. Pseudopalisades in glioblastoma are hypoxic, express extracellular matrix proteases, and are formed by an actively migrating cell population. *Cancer Res*. 2004;64(3):920-7.

[103] McMahon KM, Volpato M, Chi HY, Musiwaro P, Poterlowicz K, Peng Y, Scally AJ, Patterson LH, Phillips RM, Sutton CW. Characterization of changes in the proteome in different regions of 3D multicell tumor spheroids. *J Proteome Res*. 2012;11(5):2863-75.

[104] Hirschhaeuser F, Menne H, Dittfeld C, West J, Mueller-Klieser W, Kunz-Schughart LA. Multicellular tumor spheroids: an underestimated tool is catching up again. *J Biotechnol*. 2010;148(1):3-15.

[105] Mueller-Klieser WF, Sutherland RM. Oxygen tensions in multicell spheroids of two cell lines. *Br J Cancer*. 1982;45(2):256-64.

[106] Sutherland RM, Durand RE. Growth and cellular characteristics of multicell spheroids. Recent Results *Cancer Res*. 1984;95:24-49.

[107]Sutherland RM. Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science*. 1988;240(4849):177-84.

[108] Minchinton AI, Tannock IF. Drug penetration in solid tumours. *Nat Rev Cancer*. 2006;6(8):583-92.

[109] Kyle AH, Baker JH, Minchinton AI. Targeting quiescent tumor cells via oxygen and IGF-I supplementation. *Cancer Res*. 2012;72(3):801-9.

[110] Awale S, Lu J, Kalauni SK, Kurashima Y, Tezuka Y, Kadota S, Esumi H. Identification of arctigenin as an antitumor agent having the ability to eliminate the tolerance of cancer cells to nutrient starvation. *Cancer Res*. 2006;66(3):1751-7.

[111] Grimes DR, Kelly C, Bloch K, Partridge M. A method for estimating the oxygen consumption rate in multicellular tumour spheroids. *J R Soc Interface*. 2014;11(92):20131124.

[112] Lisanti MP, Martinez-Outschoorn UE, Chiavarina B, Pavlides S, Whitaker-Menezes D, Tsirigos A, Witkiewicz A, Lin Z, Balliet R, Howell A, Sotgia F. Understanding the "lethal" drivers of tumor-stroma co-evolution: emerging role(s) for hypoxia, oxidative stress and autophagy/mitophagy in the tumor micro-environment. *Cancer Biol Ther*. 2010;10(6):537-42.

[113] Kobayashi H, Man S, Graham CH, Kapitain SJ, Teicher BA, Kerbel RS. Acquired multicellular-mediated resistance to alkylating agents in cancer. *Proc Natl Acad Sci U S A*. 1993;90(8):3294-8.

[114] Graham CH, Kobayashi H, Stankiewicz KS, Man S, Kapitain SJ, Kerbel RS. Rapid acquisition of multicellular drug resistance after a single exposure of mammary tumor cells to antitumor alkylating agents. *J Natl Cancer Inst*. 1994;86(13):975-82.

[115] Holder JW, Elmore E, Barrett JC. Gap junction function and cancer. *Cancer Res*. 1993;53(15):3475-85.

[116] Olive PL, Durand RE. Drug and radiation resistance in spheroids: cell contact and kinetics. *Cancer Metastasis Rev*. 1994;13(2):121-38.

[117] Kunz-Schughart LA, Kreutz M, Knuechel R. Multicellular spheroids: a three-dimensional in vitro culture system to study tumour biology. *Int J Exp Pathol*. 1998;79(1):1-23.

[118] Olive PL, Chaplin DJ, Durand RE. Pharmacokinetics, binding and distribution of Hoechst 33342 in spheroids and murine tumours. *Br J Cancer*. 1985;52(5):739-46.

[119] Durand RE. Use of Hoechst 33342 for cell selection from multicell systems. *J Histochem Cytochem*. 1982;30(2):117-22.

[120] Durand RE, Olive PL. Evaluation of bioreductive drugs in multicell spheroids. *Int J Radiat Oncol Biol Phys*. 1992;22(4):689-92.

[121] Yorimitsu T, Klionsky DJ. Autophagy: molecular machinery for self-eating. *Cell Death Differ*. 2005;12 Suppl 2:1542-52.

[122] Dice JF. Chaperone-mediated autophagy. *Autophagy*. 2007;3(4):295-9.

[123] Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell*. 2004;6(4):463-77.

[124] Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science.* 2000;290(5497):1717-21.

[125] Eisenberg-Lerner A, Bialik S, Simon HU, Kimchi A. Life and death partners: apoptosis, autophagy and the cross-talk between them. *Cell Death Differ*. 2009;16(7):966-75.

[126] Gump JM, Thorburn A. Autophagy and apoptosis: what is the connection? *Trends Cell Biol*. 2011;21(7):387-92.

[127] Dorn BR, Dunn WA Jr, Progulske-Fox A. Bacterial interactions with the autophagic pathway. *Cell Microbiol*. 2002;4(1):1-10.

[128] Hernandez LD, Pypaert M, Flavell RA, Galán JE. A Salmonella protein causes macrophage cell death by inducing autophagy. *J Cell Biol*. 2003;163(5):1123-31.

[129] He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet*. 2009;43:67-93.

[130] Huang J, Klionsky DJ. Autophagy and human disease. *Cell Cycle.* 2007;6(15):1837-49.

[131] Komatsu M, Ueno T, Waguri S, Uchiyama Y, Kominami E, Tanaka K. Constitutive autophagy: vital role in clearance of unfavorable proteins in neurons. *Cell Death Differ*. 2007;14(5):887-94.

[132] Mathew R, White E. Autophagy, stress, and cancer metabolism: what doesn't kill you makes you stronger. *Cold Spring Harb Symp Quant Biol*. 2011;76:389-96.

[133] Klionsky DJ, Cregg JM, Dunn WA Jr, Emr SD, Sakai Y, Sandoval IV, Sibirny A, Subramani S, Thumm M, Veenhuis M, Ohsumi Y. A unified nomenclature for yeast autophagy-related genes. *Dev Cell*. 2003;5(4):539-45.

[134] Behrends C, Sowa ME, Gygi SP, Harper JW. Network organization of the human autophagy system. *Nature*. 2010;466(7302):68-76.

[135] Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, Lippincott-Schwartz J. Mitochondria supply membranes for autophagosome biogenesis during starvation. *Cell*. 2010;141(4):656-67.

[136] Amaravadi RK, Lippincott-Schwartz J, Yin XM, Weiss WA, Takebe N, Timmer W, DiPaola RS, Lotze MT, White E. Principles and current strategies for targeting autophagy for cancer treatment. *Clin Cancer Res*. 2011;17(4):654-66.

[137] He C, Klionsky DJ.Annu. Regulation mechanisms and signaling pathways of autophagy. *Rev Genet*. 2009;43:67-93.

[138] Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M, Kondo S, Dumont DJ, Gutterman JU, Walker CL, Slingerland JM, Mills GB. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. *Nat Cell Biol*. 2007;9(2):218-24.

[139] Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol*. 2011;13(2):132-41.

[140] Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol*. 2011;13(2):132-41.

[141] Dewaele M, Maes H, Agostinis P. ROS-mediated mechanisms of autophagy stimulation and their relevance in cancer therapy. *Autophagy*. 2010;6(7):838-54

[142] Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, Elazar Z. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *EMBO J*. 2007;26(7):1749-60.

[143] Pursiheimo JP, Rantanen K, Heikkinen PT, Johansen T, Jaakkola PM. Hypoxia-activated autophagy accelerates degradation of SQSTM1/p62. *Oncogene* 2009;28:334-44.

[144] Zhang H, Bosch-Marce M, Shimoda LA, Tan YS, Baek JH, Wesley JB, Gonzalez FJ, Semenza GL. Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *J Biol Chem* 2008; 283:10892-903.

[145] Song J, Qu Z, Guo X, Zhao Q, Zhao X, Gao L, Sun K, Shen F, Wu M, Wei L. Hypoxia-induced autophagy contributes to the chemoresistance of hepatocellular carcinoma cells. *Autophagy*. 2009;5(8):1131-44.

[146] Papandreou I, Lim AL, Laderoute K, Denko NC. Hypoxia signals autophagy in tumor cells via AMPK activity, independent of HIF-1, BNIP3, and BNIP3L. Cell Death Differ. 2008;15(10):1572-81.

[147] Song J, Qu Z, Guo X, Zhao Q, Zhao X, Gao L, Sun K, Shen F, Wu M, Wei L. Hypoxia-induced autophagy contributes to the chemoresistance of hepatocellular carcinoma cells. *Autophagy*. 2009;5(8):1131-44.

[148] Mazure NM, Pouysségur J. Hypoxia-induced autophagy: cell death or cell survival? *J.Curr Opin Cell Biol.* 2010;22(2):177-80.

[149] Papandreou I, Lim AL, Laderoute K, Denko NC. Hypoxia signals autophagy in tumor cells via AMPK activity, independent of HIF-1, BNIP3, and BNIP3L. Cell Death Differ. 2008;15(10):1572-81.

[150] Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gélinas C, Fan Y, Nelson DA, Jin S, White E. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell.* 2006;10(1):51-64.

[151] Fujii S, Mitsunaga S, Yamazaki M, Hasebe T, Ishii G, Kojima M, Kinoshita T, Ueno T, Esumi H, Ochiai A. Autophagy is activated in pancreatic cancer cells and correlates with poor patient outcome. *Cancer Sci*. 2008;99(9):1813-9.

[152] Yang S, Wang X, Contino G, Liesa M, Sahin E, Ying H, Bause A, Li Y, Stommel JM, Dell'antonio G, Mautner J, Tonon G, Haigis M, Shirihai OS, Doglioni C, Bardeesy N, Kimmelman AC. Pancreatic cancers require autophagy for tumor growth. *Genes Dev*. 2011;25(7):717-29.

[153] Amaravadi RK, Lippincott-Schwartz J, Yin XM, Weiss WA, Takebe N, Timmer W, DiPaola RS, Lotze MT, White E. Principles and current strategies for targeting autophagy for cancer treatment. *Clin Cancer Res*. 2011;17(4):654-66.

[154] Tsuchihara K, Fujii S, Esumi H.Autophagy and cancer: dynamism of the metabolism of tumor cells and tissues. *Cancer Lett*. 2009;278(2):130-8.

[155] Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, Chen G, Jin S, White E. Autophagy suppresses tumor progression by limiting chromosomal instability. *Genes Dev*. 2007;21(11):1367-81.

[156] Eisenberg-Lerner A, Kimchi A. The paradox of autophagy and its implication in cancer etiology and therapy. *Apoptosis*. 2009;14(4):376-91.

[157] Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest.* 2003;112(12):1809-20.

[158] Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest*. 2003;112(12):1809-20.

[159] Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, Chen G, Jin S, White E. Autophagy suppresses tumor progression by limiting chromosomal instability. *Genes Dev*. 2007;21(11):1367-81.

[160] Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gélinas C, Fan Y, Nelson DA, Jin S, White E. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell*. 2006;10(1):51-64.

[161] Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, Chen G, Jin S, White E. Autophagy suppresses tumor progression by limiting chromosomal instability. *Genes Dev*. 2007;21(11):1367-81.

[162] Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gélinas C, Fan Y, Nelson DA, Jin S, White E. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell*. 2006;10(1):51-64.

[163] Martinez-Outschoorn UE, Balliet RM, Rivadeneira DB, Chiavarina B, Pavlides S, Wang C, Whitaker-Menezes D, Daumer KM, Lin Z, Witkiewicz AK, Flomenberg N, Howell A, Pestell RG, Knudsen ES, Sotgia F, Lisanti MP. Oxidative stress in cancer associated fibroblasts drives tumor-stroma co-evolution: A new paradigm for understanding tumor metabolism, the field effect and genomic instability in cancer cells. *Cell Cycle*. 2010;9(16):3256-76.

[164] Martinez-Outschoorn UE, Balliet RM, Rivadeneira DB, Chiavarina B, Pavlides S, Wang C, Whitaker-Menezes D, Daumer KM, Lin Z, Witkiewicz AK, Flomenberg N, Howell A, Pestell RG, Knudsen ES, Sotgia F, Lisanti MP. Oxidative stress in cancer associated fibroblasts drives tumor-stroma co-evolution: A new paradigm for understanding tumor metabolism, the field effect and genomic instability in cancer cells. *Cell Cycle*. 2010;9(16):3256-76.

[165] Djavaheri-Mergny M, Amelotti M, Mathieu J, Besançon F, Bauvy C, Souquère S, Pierron G, Codogno P. NF-kappaB activation represses tumor necrosis factor-alpha-induced autophagy. *J Biol Chem*. 2006;281(41):30373-82.

[166] Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science*. 2004;304(5676):1500-2.

[167] Feng Z, Zhang H, Levine AJ, Jin S. The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci U S A*. 2005;102(23):8204-9.

[168] Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, Gasco M, Garrone O, Crook T, Ryan KM. DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. *Cell*. 2006;126(1):121-34.

[169] Tasdemir E, Maiuri MC, Galluzzi L, Vitale I, Djavaheri-Mergny M, D'Amelio M, Criollo A, Morselli E, Zhu C, Harper F, Nannmark U, Samara C, Pinton P, Vicencio JM, Carnuccio R, Moll UM, Madeo F, Paterlini-Brechot P, Rizzuto R, Szabadkai G, Pierron G, Blomgren K, Tavernarakis N, Codogno P, Cecconi F, Kroemer G. Regulation of autophagy by cytoplasmic p53. *Nat Cell Biol*. 2008;10(6):676-87.

[170]Kurt Degenhardt, Robin Mathew, Brian Beaudoin, Kevin Bray, Diana Anderson, Guanghua Chen, Chandreyee Mukherjee, Yufang Shi, Céline Gélinas, Yongjun Fan, Deirdre A. Nelson, Shengkan Jin, Eileen White. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell*. 2006; 10(1): 51–64.

[171] Tanida I, Minematsu-Ikeguchi N, Ueno T, Komi nami E. Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. *Autophagy*. 2005;1(2):84-91.

[172]Klionsky DJ et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy*. 2008;4(2):151-75.

[173] Valentijn AJ, Zouq N, Gilmore AP. Anoikis. *Biochem Soc Trans*. 2004;32(Pt3):421-5.

[174] Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol*. 1994;124(4):619-26.

[175] Chiarugi P, Giannoni E. Anoikis: a necessary death program for anchorage-dependent cells. *Biochem Pharmacol*. 2008;76(11):1352-64.

[176] Guadamillas MC, Cerezo A, Del Pozo MA. Overcoming anoikis--pathways to anchorage-independent growth in cancer. *J Cell Sci*. 2011;124(Pt 19):3189-97.

[177] Chiarugi P, Giannoni E. Anoikis: a necessary death program for anchorage-dependent cells. *Biochem Pharmacol*. 2008;76(11):1352-64.

[178] Yawata A, Adachi M, Okuda H, Naishiro Y, Takamura T, Hareyama M, Takayama S, Reed JC, Imai K. Prolonged cell survival enhances peritoneal dissemination of gastric cancer cells. *Oncogene*. 1998;16(20):2681-6.

[179] Shanmugathasan M, Jothy S. Apoptosis, anoikis and their relevance to the pathobiology of colon cancer. *Pathol Int*. 2000;50(4):273-9.

[180] Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol*. 1994;124(4):619-26.

[181] Frisch SM, Schaller M, Cieply B. Mechanisms that link the oncogenic epithelial-mesenchymal transition to suppression of anoikis. *J Cell Sci*. 2013;126(Pt 1):21-9.

[182] Smit MA, Geiger TR, Song JY, Gitelman I, Peeper DS. A Twist-Snail axis critical for TrkB-induced epithelial-mesenchymal transition-like transformation, anoikis resistance, and metastasis. *Mol Cell Biol*. 2009;29(13):3722-37.

[183] Guo W, Giancotti FG. Integrin signalling during tumour progression. *Nat Rev Mol Cell Biol*. 2004;5(10):816-26.

[184] Plantefaber LC, Hynes RO. Changes in integrin receptors on oncogenically transformed cells. *Cell.* 1989;56(2):281-90.

[185] Frisch SM, Screaton RA. Anoikis mechanisms. *Curr Opin Cell Biol*. 2001;13(5):555-62.

[186] Tsuji T, Ibaragi S, Hu GF. Epithelial-mesenchymal transition and cell cooperativity in metastasis. *Cancer Res*. 2009;69(18):7135-9.

[187] Kanayama S, Yamada Y, Kawaguchi R, Tsuji Y, Haruta S, Kobayashi H. Hepatocyte growth factor induces anoikis resistance by up-regulation of cyclooxygenase-2 expression in uterine endometrial cancer cells. *Oncol Rep*. 2008;19(1):117-22.

[188] Guadamillas MC, Cerezo A, Del Pozo MA. Overcoming anoikis--pathways to anchorage-independent growth in cancer. *J Cell Sci*. 2011;124(Pt 19):3189-97.

[189] Cerezo A, Guadamillas MC, Goetz JG, Sánchez-Perales S, Klein E, Assoian RK, del Pozo MA. The absence of caveolin-1 increases proliferation and anchorage-independent growth by a Rac-dependent, Erk-independent mechanism. *Mol Cell Biol*. 2009;29(18):5046-59.

[190] Fung C, Lock R, Gao S, Salas E, Debnath J. Induction of autophagy during extracellular matrix detachment promotes cell survival. *Mol Biol Cell*. 2008;19(3):797-806.

[191] Debnath J. Detachment-induced autophagy in three-dimensional epithelial cell cultures. Methods Enzymol. 2009;452:423-39.

[192] Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res*. 1961;25:585-621.

[193] Ewald JA, Desotelle JA, Wilding G, Jarrard DF. Therapy-induced senescence in cancer. *J Natl Cancer Inst*. 2010;102(20):1536-46.

[194] Campisi J. Aging, cellular senescence, and cancer. *Annu Rev Physiol*. 2013;75:685-705.

[195] Ewald JA, Desotelle JA, Wilding G, Jarrard DF. Therapy-induced senescence in cancer. *J Natl Cancer Inst*. 2010;102(20):1536-46.

[196] Campisi J. Aging, cellular senescence, and cancer. *Annu Rev Physiol*. 2013;75:685-705.

[197] Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, Majoor DM, Shay JW, Mooi WJ, Peeper DS. BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature*. 2005;436(7051):720-4.

[198] Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M, Benguría A, Zaballos A, Flores JM, Barbacid M, Beach D, Serrano M. Tumour biology: senescence in premalignant tumours. *Nature*. 2005;436(7051):642.

[199] Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100(1):57-70.

[200] Chang BD, Broude EV, Dokmanovic M, Zhu H, Ruth A, Xuan Y, Kandel ES, Lausch E, Christov K, Roninson IB.
A senescence-like phenotype distinguishes tumor cells that undergo terminal proliferation arrest after exposure to anticancer agents. *Cancer Res*. 1999;59(15):3761-7.

[201] Hwang ES. Replicative senescence and senescence-like state induced in cancer-derived cells. *Mech Ageing Dev*. 2002;123(12):1681-94.

[202] Gewirtz DA, Holt SE, Elmore LW. Accelerated senescence: an emerging role in tumor cell response to chemotherapy and radiation. *Biochem Pharmacol*. 2008;76(8):947–957.

[203] Schmitt CA, Fridman JS, Yang M, Lee S, Baranov E, Hoffman RM, Lowe SW. A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. *Cell*. 2002;109(3):335-46.

[204] Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O.
A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A*. 1995;92(20):9363-7.

[205] Zhang R, Chen W, Adams PD. Molecular dissection of formation of senescence-associated heterochromatin foci. *Mol Cell Biol*. 2007;27(6):2343-58.

[206] Ewald JA, Desotelle JA, Wilding G, Jarrard DF. Therapy-induced senescence in cancer. *J Natl Cancer Inst*. 2010;102(20):1536-46.

[207] Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem*. 1998;273(10):5858-68.

[208] Shiloh Y. The ATM-mediated DNA-damage response: taking shape. *Trends Biochem Sci*. 2006;31(7):402-10.

[209] Wang C, Jurk D, Maddick M, Nelson G, Martin-Ruiz C, von Zglinicki T. DNA damage response and cellular senescence in tissues of aging mice. *Aging Cell*. 2009;8(3):311-23.

[210] Lawless C, Wang C, Jurk D, Merz A, Zglinicki Tv, Passos JF. Quantitative assessment of markers for cell senescence. *Exp Gerontol*. 2010;45(10):772-8.

[211] te Poele RH, Okorokov AL, Jardine L, Cummings J, Joel SP. DNA damage is able to induce senescence in tumor cells in vitro and in vivo. *Cancer Res*. 2002;62(6):1876-83.

[212] Dilley TK, Bowden GT, Chen QM. Novel mechanisms of sublethal oxidant toxicity: induction of premature senescence in human fibroblasts confers tumor promoter activity. *Exp Cell Res*. 2003;290(1):38-48.

[213] Krtolica A, Parrinello S, Lockett S, Desprez PY, Campisi J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci U S A*. 2001;98(21):12072-7.

[214] Roberson RS, Kussick SJ, Vallieres E, Chen SY, Wu DY. Escape from therapy-induced accelerated cellular senescence in p53-null lung cancer cells and in human lung cancers. *Cancer Res*. 2005;65(7):2795-803.

[215] Wang Q, Wu PC, Roberson RS, Luk BV, Ivanova I, Chu E, Wu DY. Survivin and escaping in therapy-induced cellular senescence. *Int J Cancer*. 2011;128(7):1546-58.

[216]Ansieau S, Bastid J, Doreau A, Morel AP, Bouchet BP, Thomas C, Fauvet F, Puisieux I, Doglioni C, Piccinin S, Maestro R, Voeltzel T, Selmi A, Valsesia-Wittmann S, Caron de Fromentel C, Puisieux A. Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence. *Cancer Cell*. 2008;14(1):79-89.

[217] Young AR, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JF, Tavaré S, Arakawa S, Shimizu S, Watt FM, Narita M. Autophagy mediates the mitotic senescence transition. *Genes Dev*. 2009;23(7):798-803.

[218] Patschan S, Chen J, Polotskaia A, Mendelev N, Cheng J, Patschan D, Goligorsky MS. Lipid mediators of autophagy in stress-induced premature senescence of endothelial cells. *Am J Physiol Heart Circ Physiol*. 2008;294(3):H1119-29.

[219] Pantel K, Brakenhoff RH. Dissecting the metastatic cascade. *Nat Rev Cancer*. 2004;4(6):448-56.

[220] Rupert A Willis (1952). The Spread of Tumours in the Human Body. London: Butterworth and Co.

[221] Karrison TG, Ferguson DJ, Meier P. Dormancy of mammary carcinoma after mastectomy. *J Natl Cancer Inst*. 1999;91(1):80-5.

[222] Weckermann D, Müller P, Wawroschek F, Harzmann R, Riethmüller G, Schlimok G. Disseminated cytokeratin positive tumor cells in the bone marrow of

patients with prostate cancer: detection and prognostic value. *J Urol*. 2001;166(2):699-703.

[223] Aguirre-Ghiso JA. Models, mechanisms and clinical evidence for cancer dormancy. *Nat Rev Cancer*. 2007;7(11):834-46.

[224] Aguirre-Ghiso JA. Models, mechanisms and clinical evidence for cancer dormancy. Nat Rev Cancer. 2007;7(11):834-4.

[225] Roninson IB. Tumor cell senescence in cancer treatment. *Cancer Res*. 2003;63(11):2705-15.

[226] Wells A1, Griffith L, Wells JZ, Taylor DP.The dormancy dilemma: quiescence versus balanced proliferation. Cancer Res. 2013;73(13):3811-6.

[227] Zhang J1, Grindley JC, Yin T, Jayasinghe S, He XC, Ross JT, Haug JS, Rupp D, Porter-Westpfahl KS, Wiedemann LM, Wu H, Li L. PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature*. 2006;441(7092):518-22.

[228] Yokota J. Tumor progression and metastasis. *Carcinogenesis*. 2000;21(3):497-503.

[229] Demicheli R. Tumour dormancy: findings and hypotheses from clinical research on breast cancer. Semin *Cancer Biol*. 2001;11(4):297-306.

[230] Boudreau N1, Bissell MJ. Extracellular matrix signaling: integration of form and function in normal and malignant cells. *Curr Opin Cell Biol.* 1998;10(5):640-6.

[231] Aguirre Ghiso JA1, Kovalski K, Ossowski L. Tumor dormancy induced by downregulation of urokinase receptor in human carcinoma involves integrin and MAPK signaling. *J Cell Biol.* 1999;147(1):89-104.

[232] Naumov GN1, Akslen LA, Folkman J. Role of angiogenesis in human tumor dormancy: animal models of the angiogenic switch. *Cell Cycle*. 2006;5(16):1779-87.

[233] Naumov GN1, Townson JL, MacDonald IC, Wilson SM, Bramwell VH, Groom AC, Chambers AF. Ineffectiveness of doxorubicin treatment on solitary dormant mammary carcinoma cells or late-developing metastases. *Breast Cancer Res Treat*. 2003;82(3):199-206.

[234] Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell*. 2008;14(6):818-29.

[235] Nelson WJ. Remodeling epithelial cell organization: transitions between front-rear and apical-basal polarity. *Cold Spring Harb Perspect Biol*. 2009;1(1):a000513.

[236] Greenburg G, Hay ED. Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. J Cell Biol. 1982 Oct;95(1):333-9.

[237] Nelson WJ. Remodeling epithelial cell organization: transitions between front-rear and apical-basal polarity. *Cold Spring Harb Perspect Biol*. 2009;1(1):a000513.

[238] Guarino M. Epithelial-to-mesenchymal change of differentiation. From embryogenetic mechanism to pathological patterns. *Histol Histopathol*. 1995;10(1):171-84.

[239] Chaffer CL, Thompson EW, Williams ED. Mesenchymal to epithelial transition in development and disease. *Cells Tissues Organs*. 2007;185(1-3):7-19.

[240] Zuk A, Hay ED. Expression of beta 1 integrins changes during transformation of avian lens epithelium to mesenchyme in collagen gels. *Dev Dyn*. 1994;201(4):378-93.

[241] Savagner P, Vallés AM, Jouanneau J, Yamada KM, Thiery JP. Alternative splicing in fibroblast growth factor receptor 2 is associated with induced epithelial-mesenchymal transition in rat bladder carcinoma cells. *Mol Biol Cell*. 1994;5(8):851-62.

[242] Bilder D. Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. *Genes Dev*. 2004;18(16):1909-25.

[243] Boyer B, Thiery JP. Epithelium-mesenchyme interconversion as example of epithelial plasticity. *APMIS*. 1993;101(4):257-68.

[244] Burdsal CA, Damsky CH, Pedersen RA. The role of E-cadherin and integrins in mesoderm differentiation and migration at the mammalian primitive streak. *Development*. 1993;118(3):829-44.

[245] Guilford P. E-cadherin downregulation in cancer: fuel on the fire? *Mol Med Today*. 1999;5(4):172-7.

[246]Cano A, Pérez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol*. 2000;2(2):76-83.

[247] Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, Savagner P, Gitelman I, Richardson A, Weinberg RA.bTwist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell*. 2004;117(7):927-39.

[248] Magee JA, Piskounova E, Morrison SJ. Cancer stem cells: impact, heterogeneity, and uncertainty. *Cancer Cell*. 2012;21(3):283-96.

[249] Sampieri K, Fodde R. Cancer stem cells and metastasis. *Semin Cancer Biol*. 2012;22(3):187-93.

[250] Brabletz T. EMT and MET in metastasis: where are the cancer stem cells? *Cancer Cell*. 2012;22(6):699-701.

[251] Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell*. 1990;61(5):759-67.

[252] Puglisi MA, Sgambato A, Saulnier N, Rafanelli F, Barba M, Boninsegna A, Piscaglia AC, Lauritano C, Novi ML, Barbaro F, Rinninella E, Campanale C, Giuliante F, Nuzzo G, Alfieri S, Doglietto GB, Cittadini A, Gasbarrini A. Isolation and characterization of CD133+ cell population within human primary and metastatic colon cancer. *Eur Rev Med Pharmacol Sci*. 2009;13 Suppl 1:55-62.

[253] Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature*. 2001;414(6859):105-11.

[254] Polakis P. Wnt signaling and cancer. *Genes Dev*. 2000;14(15):1837-51.

[255] Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A*. 2003;100(7):3983-8.

[256] Reya T, Clevers H. Wnt signalling in stem cells and cancer. *Nature*. 2005;434(7035):843-50.

[257] Kemper K, Prasetyanti PR, De Lau W, Rodermond H, Clevers H, Medema JP. Monoclonal antibodies against Lgr5 identify human colorectal cancer stem cells. *Stem Cells*. 2012;30(11):2378-86

[258] Van der Flier LG, Sabates-Bellver J, Oving I, Haegebarth A, De Palo M, Anti M, Van Gijn ME, Suijkerbuijk S, Van de Wetering M, Marra G, Clevers H. The Intestinal Wnt/TCF Signature. *Gastroenterology*. 2007;132(2):628-32.

[259] Potten CS, Kellett M, Rew DA, Roberts SA. Proliferation in human gastrointestinal epithelium using bromodeoxyuridine in vivo: data for different sites, proximity to a tumour, and polyposis coli. *Gut*. 1992;33(4):524-9.

[260] Fevr T, Robine S, Louvard D, Huelsken J. Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. *Mol Cell Biol*. 2007;27(21):7551-9.

[261] Puglisi MA, Tesori V, Lattanzi W, Gasbarrini GB, Gasbarrini A. Colon cancer stem cells: controversies and perspectives. *World J Gastroenterol*. 2013;19(20):2997-3006.

[262] Shih IM, Wang TL, Traverso G, Romans K, Hamilton SR, Ben-Sasson S, Kinzler KW, Vogelstein B. Top-down morphogenesis of colorectal tumors. *Proc Natl Acad Sci U S A*. 2001;98(5):2640-5.

[263] Barker N, Ridgway RA, van Es JH, van de Wetering M, Begthel H, van den Born M, Danenberg E, Clarke AR, Sansom OJ, Clevers H. Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature*. 2009;457(7229):608-11.

[264] Shih IM, Wang TL, Traverso G, Romans K, Hamilton SR, Ben-Sasson S, Kinzler KW, Vogelstein B. Top-down morphogenesis of colorectal tumors. *Proc Natl Acad Sci U S A*. 2001;98(5):2640-5.

[265] Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL, Wahl GM. Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res*. 2006;66(19):9339-44.

[266]Wicha MS, Liu S, Dontu G. Cancer stem cells: an old idea--a paradigm shift. *Cancer Res*. 2006;66(4):1883-90; discussion 1895-6.

[267]Moore N, Lyle S. Quiescent, slow-cycling stem cell populations in cancer: a review of the evidence and discussion of significance. *J Oncol*. 2011;2011. pii: 396076.

[268] Viale A, Pelicci PG. Awaking stem cells from dormancy: growing old and fighting cancer. *EMBO Mol Med*. 2009;1(2):88-91.

[269] Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer*. 2005;5:275-84.

[270] Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature*. 2006;444(7120):756-60.

[271] Eyler CE, Rich JN. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. *J Clin Oncol*. 2008;26(17):2839-45.

[272] Woodward WA, Chen MS, Behbod F, Alfaro MP, Buchholz TA, Rosen JM. WNT/beta-catenin mediates radiation resistance of mouse mammary progenitor cells. *Proc Natl Acad Sci U S A*. 2007;104(2):618-23.

[273] Merritt AJ, Potten CS, Watson AJM, et al. Differential expression of bcl-2 in intestinal epithelia. Correlation with attenuation of apoptosis in colonic crypts and the incidence of colonic neoplasia. *Journal of Cell Science*. 1995;108, part 6:2261–2271.

[274] Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, Tripodo C, Russo A, Gulotta G, Medema JP, Stassi G. Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell Stem Cell*. 2007;1(4):389-402.

[275] Todaro M, Perez Alea M, Scopelliti A, Medema JP, Stassi G. IL-4-mediated drug resistance in colon cancer stem cells. *Cell Cycle*. 2008;7(3):309-13.

[276] Moreb JS. Aldehyde dehydrogenase as a marker for stem cells. *Curr Stem Cell Res Ther*. 2008;3(4):237-46.

[277] Magni M, Shammah S, Schiró R, Mellado W, Dalla-Favera R, Gianni AM. Induction of cyclophosphamide-resistance by aldehyde-dehydrogenase gene transfer. *Blood*. 1996;87(3):1097-103.

[278] Pearce DJ, Taussig D, Simpson C, Allen K, Rohatiner AZ, Lister TA, Bonnet D. Characterization of cells with a high aldehyde dehydrogenase activity from cord blood and acute myeloid leukemia samples. *Stem Cells*. 2005;23(6):752-60.

[279] Eyler CE, Rich JN. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. *J Clin Oncol*. 2008;26(17):2839-45.

[280]Moore N, Lyle S. Quiescent, slow-cycling stem cell populations in cancer: a review of the evidence and discussion of significance. *J Oncol*. 2011;2011. pii: 396076.

[281] Huang EH, Hynes MJ, Zhang T, Ginestier C, Dontu G, Appelman H, Fields JZ, Wicha MS, Boman BM.

Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. *Cancer Res*. 2009;69(8):3382-9.

[282] Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, Hoey T, Gurney A, Huang EH, Simeone DM, Shelton AA, Parmiani G, Castelli C, Clarke MF. Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci U S A*. 2007;104(24):10158-63.

[283] Naor D, Nedvetzki S, Golan I, Melnik L, Faitelson Y. CD44 in cancer. *Crit Rev Clin Lab Sci.* 2002;39(6):527-79.

[284] Yang ZF, Ngai P, Ho DW, Yu WC, Ng MN, Lau CK, Li ML, Tam KH, Lam CT, Poon RT, Fan ST. Identification of local and circulating cancer stem cells in human liver cancer. *Hepatology*. 2008;47(3):919-28.

[285] Zeilstra J, Joosten SP, Dokter M, Verwiel E, Spaargaren M, Pals ST. Deletion of the WNT target and cancer stem cell marker CD44 in Apc(Min/+) mice attenuates intestinal tumorigenesis. *Cancer Res*. 2008;68(10):
3655-61.

[286] O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature*. 2007;445(7123):106-10.

[287] Choi D, Lee HW, Hur KY, Kim JJ, Park GS, Jang SH, Song YS, Jang KS, Paik SS. Cancer stem cell markers CD133 and CD24 correlate with invasiveness and differentiation in colorectal adenocarcinoma. *World J Gastroenterol*. 2009;15(18):2258-64.

[288] Ke J, Wu X, Wu X, He X, Lian L, Zou Y, He X, Wang H, Luo Y, Wang L, Lan P. A subpopulation of $CD24^{+}$ cells in colon cancer cell lines possess stem cell characteristics. *Neoplasma*. 2012;59(3):282-8.

[289]Yeung TM, Gandhi SC, Wilding JL, Muschel R, Bodmer WF. Cancer stem cells from colorectal cancer-derived cell lines. *Proc Natl Acad Sci U S A*. 2010;107(8):3722-7.

[290] Schofield, R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells*. 1978;4(1-2):7-25.

[291] Dick JE. Stem cell concepts renew cancer research. *Blood*. 2008;112(13):4793-807.

[292] Li L, Neaves WB. Normal stem cells and cancer stem cells: the niche matters. *Cancer Res*. 2006;66(9):4553-7.

[293] Gottardi CJ, Gumbiner BM. Distinct molecular forms of beta-catenin are targeted to adhesive or transcriptional complexes. *J Cell Biol*. 2004;167(2):339-49.

[294] Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, Crystal RG, Besmer P, Lyden D, Moore MA, Werb Z, Rafii S. Recruitment of stem and

progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell*. 2002;109(5):625-37.

[295] Curran S, Murray GI. Matrix metalloproteinases in tumour invasion and metastasis. *J Pathol*. 1999; 189(3):300-8.

[296] Hirsch E, Iglesias A, Potocnik AJ, Hartmann U, Fässler R. Impaired migration but not differentiation of haematopoietic stem cells in the absence of beta1 integrins. *Nature*. 1996;380(6570):171-5.

[297] Andressen C, Arnhold S, Puschmann M, Bloch W, Hescheler J, Fässler R, Addicks K. Beta1 integrin deficiency impairs migration and differentiation of mouse embryonic stem cell derived neurons. *Neurosci Lett*. 1998;251(3):165-8.

[298] Missan DS, DiPersio M. Integrin control of tumor invasion. *Crit Rev Eukaryot Gene Expr*. 2012;22(4):309-24.

[299] Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. *Nature.* 2001;414(6859):98-104.

[300] Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. *Science*. 2011;331(6024):1559-64.

[301] Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer*. 2002;2(6):442-54.

[302] Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol*. 2003;15(6):740-6.

[303] Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A*. 2003;100(7):3983-8.

[304] Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*. 2008;133(4):704-15.

[305] Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ, Heeschen C. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell.* 2007;1(3):313-23.

[306] Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat Rev Cancer*. 2005;5(9):744-9.

[307] Gazzaniga P, Gradilone A, Petracca A, Nicolazzo C, Raimondi C, Iacovelli R, Naso G, Cortesi E. Molecular markers in circulating tumour cells from metastatic colorectal cancer patients. *J Cell Mol Med*. 2010;14(8):2073-7

[308] Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat Rev Cancer*. 2005;5(9):744-9.

[309] Ueno H, Murphy J, Jass JR, Mochizuki H, Talbot IC. Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. *Histopathology*. 2002;40(2):127-32.

[310] Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat Rev Cancer*. 2005;5(9):744-9.

[311] Rausch V, Liu L, Apel A, Rettig T, Gladkich J, Labsch S, Kallifatidis G, Kaczorowski A, Groth A, Gross W, Gebhard MM, Schemmer P, Werner J, Salnikov AV, Zentgraf H, Büchler MW, Herr I. Autophagy mediates survival of pancreatic tumour-initiating cells in a hypoxic microenvironment. *J Pathol*. 2012;227(3):325-35.

[312] Song YJ, Zhang SS, Guo XL, Sun K, Han ZP, Li R, Zhao QD, Deng WJ, Xie XQ, Zhang JW, Wu MC, Wei LX. Autophagy contributes to the survival of CD133+ liver cancer stem cells in the hypoxic and nutrient-deprived tumor microenvironment. *Cancer Lett*. 2013;339(1):70-81.

[313]Phadwal K, Watson AS, Simon AK. Tightrope act: autophagy in stem cell renewal, differentiation, proliferation, and aging. *Cell Mol Life Sci*. 2013;70(1):89-103.

[314] Chen C, Liu Y, Liu R, Ikenoue T, Guan KL, Liu Y, Zheng P. TSC-mTOR maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species. *J Exp Med*. 2008;205(10):2397-408.

[315] Wu S, Wang X, Chen J, Chen Y. Autophagy of cancer stem cells is involved with chemoresistance of colon cancer cells. *Biochem Biophys Res Commun*. 2013;434(4): 898-903.

[316] Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL, Wahl GM. Cancer stem cells--perspectives on current status and future

directions: AACR Workshop on cancer stem cells. *Cancer Res*. 2006;66(19):9339-44.

[317] Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL, Wahl GM. Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res*. 2006;66(19):9339-44.

[318] Reynolds BA, Weiss S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol*. 1996;175(1): 1-13.

[319] Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev*. 2003;17(10):1253-70.

[320] Suzuki A1, Oyama K, Fukao K, Nakauchi H, Taniguchi H. Establishment of clonal colony-forming assay system for pancreatic stem/progenitor cells. *Cell Transplant.* 2002;11(5):451-3.

[321] Shi X, Gipp J, Bushman W.Anchorage-independent culture maintains prostate stem cells. *Dev Biol*. 2007;312(1):396-406.

[322] Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA, Daidone MG. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res*. 2005;65(13):5506-11.

[323] Gibbs CP, Kukekov VG, Reith JD, Tchigrinova O, Suslov ON, Scott EW, Ghivizzani SC, Ignatova TN, Steindler DA. Stem-like cells in bone sarcomas: implications for tumorigenesis. *Neoplasia*. 2005;7(11):967-76.

[324] Fang D, Nguyen TK, Leishear K, Finko R, Kulp AN, Hotz S, Van Belle PA, Xu X, Elder DE, Herlyn M. A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer Res*. 2005;65(20):9328-37.

[325] Fábián A, Barok M, Vereb G, Szöllosi J. Die hard: are cancer stem cells the Bruce Willises of tumor biology? *Cytometry A*. 2009;75(1):67-74.

[326] Grotenhuis BA, Wijnhoven BP, van Lanschot JJ. Cancer stem cells and their potential implications for the treatment of solid tumors. *J Surg Oncol*. 2012;106(2):209-15.

[327] Dylla SJ, Beviglia L, Park IK, Chartier C, Raval J, Ngan L, Pickell K, Aguilar J, Lazetic S, Smith-Berdan S, Clarke MF, Hoey T, Lewicki J, Gurney AL. Colorectal

cancer stem cells are enriched in xenogeneic tumors following chemotherapy. *PLoS One*. 2008;3(6):e2428.

[328]Grotenhuis BA, Wijnhoven BP, van Lanschot JJ. Cancer stem cells and their potential implications for the treatment of solid tumors. *J Surg Oncol*. 2012;106(2):209-15.

[329] Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, Lander ES. Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell*. 2009;138(4):645-59.

[330] Jin L, Hope KJ, Zhai Q, Smadja-Joffe F, Dick JE.. Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nat Med*. 2006;12(10):1167-74.

[331] Frank NY, Margaryan A, Huang Y, Schatton T, Waaga-Gasser AM, Gasser M, Sayegh MH, Sadee W, Frank MH. ABCB5-mediated doxorubicin transport and chemoresistance in human malignant melanoma. *Cancer Res*. 2005;65(10):4320-33.

[332] Todaro M, Lombardo Y, Francipane MG, Alea MP, Cammareri P, Iovino F, Di Stefano AB, Di Bernardo C, Agrusa A, Condorelli G, Walczak H, Stassi G. Apoptosis resistance in epithelial tumors is mediated by tumor-cell-derived interleukin-4. *Cell Death Differ*. 2008;15(4):762-72.

[333] van Es JH, Clevers H. Notch and Wnt inhibitors as potential new drugs for intestinal neoplastic disease. *Trends Mol Med*. 2005;11(11):496-502.

[334] McMahon KM, Volpato M, Chi HY, Musiwaro P, Poterlowicz K, Peng Y, Scally AJ, Patterson LH, Phillips RM, Sutton CW. Characterization of changes in the proteome in different regions of 3D multicell tumor spheroids. *J Proteome Res.* 2012;11(5):2863-75.

[335] Vakkila J, Lotze MT. Inflammation and necrosis promote tumour growth. *Nat Rev Immunol*. 2004;4(8):641-8.

[336] Edwards JG, Swinson DE, Jones JL, Muller S, Waller DA, O'Byrne KJ: Tumor necrosis correlates with angiogenesis and is a predictor of poor prognosis in malignant mesothelioma. *Chest*. 2003;124: 1916-1923.

[337] Langner C, Hutterer G, Chromecki T, Leibl S, Rehak P, Zigeuner R: Tumor necrosis as prognostic indicator in transitional cell carcinoma of the upper urinary tract. *J Urol*. 2006;176: 910-914.

[338] McMahon KM, Volpato M, Chi HY, Musiwaro P, Poterlowicz K, Peng Y, Scally AJ, Patterson LH, Phillips RM, Sutton CW. Characterization of changes in the

proteome in different regions of 3D multicell tumor spheroids. *J Proteome Res*. 2012;11(5):2863-75.

Chapter 1: Identification of viable cells within the Necrotic Core of multicellular tumour spheroids (MCTS).

1 Introduction

The first experimental chapter in this thesis is concerned with whether there is any 'life' within the necrotic core of MCTS and if this can be characterised. As described previously tumour necrosis has long been associated with poor prognosis yet the mechanisms behind this association have yet to be understood. However active enzyme activity recently discovered in the necrotic core of MCTS[1] has led to the formation of a new hypothesis to explain the link. If viable cells are able to survive within areas of tumour necrosis and return to proliferation when conditions improve then this could explain the association and lead to the discovery of new biology and targets for cancer therapy. The tumour microenvironment which is known to act as a niche for different populations of cells with different phenotypes is considered a valid target for chemotherapeutic intervention however most research involves the hypoxic or tumour stoma microenvironment. The necrotic tumour microenvironment has been largely ignored due to the assumption that it consists of only dead cells. Autophagy is an evolutionarily conserved cellular response to low nutrient and oxygen conditions, such as those seen in areas of necrosis in MCTS. The role of autophagy in tumour development and progression is a paradoxical one with roles being uncovered in both tumour promoting and tumour inhibiting mechanisms. The discovery of full length proteins involved in autophagy in the necrotic core of MCTS[2] prompts further investigation in autophagy within MCTS. The specific aims of this chapter are to identify, characterise and isolate the living cells within the necrotic core of MCTS.

2 Materials and Methods

2.1 Cell Lines

DLD-1 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI 1640 medium (ATCC, Manassas, VA, USA) supplemented with heat inactivated Foetal Bovine Serum (FBS) (Invitrogen, Camarillo, CA, USA) to a final concentration of 10% (v/v). HT-29 cells were obtained from American Type Culture Collection and maintained in McCoy's 5A Medium Modified (ATCC, Manassas, VA, USA) supplemented with heat inactivated FBS to give a final concentration of 10% (v/v). MCF-7 cells were obtained from American Type Culture Collection and maintained in Eagle's Minimum Essential Medium (ATCC, Manassas, VA, USA) supplemented with 0.01 mg/ml Bovine Insulin (Sigma Aldrich, St Louis, MO, USA) and heat inactivated FBS to give a final concentration of 10% (v/v). HCT 116 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium (Life Technologies, ThermoFisherScientific, MA, USA) supplemented with heat inactivated FBS to give a final concentration of 10% (v/v).

2.2 Routine Cell Culture

All cells were incubated at 37^{o}C in 95% air, 5% CO_2 in T75 flasks until approximately 90% confluence and then passaged. Monolayers were washed twice in 10ml of Hanks Balanced Salt Solution (Sigma Aldrich, St. Louis, MO, USA) before incubation at 37^{o}C in 0.25% (w/v) trypsin (Sigma Aldrich, St. Louis, MO, USA) for 5-15 minutes until a single cell population was obtained. Trypsin was neutralised with 10ml complete medium before the cell suspension was centrifuged at 1000*g* for 5 minutes. The cell pellet was re-suspended in 10ml of medium and then split between different T75 flasks

using a typical split ratio of 1:10. Cells were then incubated as described above and medium was changed 2-3 times a week.

2.3 HT-29 Multicell tumour spheroid (MCTS) Culture

Cells ($1x10^6$) were seeded into a spinner flask (F7690, Techne, Bibby Scientific Limited, Staffordshire, UK) containing 150ml of medium, and placed on a magnetic stirrer plate (MCS-104S, Techne, Bibby Scientific Limited, Staffordshire, UK) where the medium was stirred at a rate of 55rpm. Spheroids were left to grow for 4-5 days before the medium was first changed, thereafter medium was changed every 48 hours and then 24 hours as needed.

2.4 DLD-1MCTS Culture

Cells (5×10^5) were plated into T75 flasks that had been base coated with 1% agar and incubated in 10-20ml medium for 24-72 hours. Once small spheroids had formed, they were transferred to spinner flask and medium volume increased to 150ml as described above. Spheroids were left to grow for 4-5 days before the medium was first changed, thereafter medium was changed when needed.

2.5 HCT 116 MCTS Culture

Cells (7.5×10^3) were plated into individual 1% agar coated wells in a 96 well plate. Cells were incubated for 4-5 days in 200µl of medium in the 96 well plate until the cells had formed a MCTS. MCTSs were then individually transferred to a spinner flask for continued culture. MCTS were initially incubated in 150ml medium which was increased to 250ml as the spheroids increased in size.

2.6 MCTS Growth Curves

MCTS growth curves were generated by measuring the average diameter of MCTS. The average spheroid diameter was determined by measuring 20 spheroids every 24-48hrs

using an inverted microscope fitted with a graduated eyepiece graticule. In order to visualise the morphology of MCTS at different stages of the growth curve, spheroids were removed from the spinner flask and fixed in Bouin's Solution (Sigma Aldrich, St. Louis, MO, USA) before being processed and embedded in paraffin wax, sectioned and stained using Haematoxylin and Eosin as described elsewhere (see section 2.10).

2.7 Characterising Autophagy Inhibitors

In order to determine which autophagy inhibitor was the best to use for assessing autophagic flux, Bafilomycin A1 (10nM, Enzo Life Sciences, New York, USA), chloroquine (50mM, Sigma Aldrich, St. Louis, MO, USA), and 3-methylalanine (5mM, Sigma Aldrich, St. Louis, MO, USA) were all tested on starved and un-starved cells to see their effects. Briefly, $2x10^4$ cells were plated on coverslips in 6-well plates and grown under normal culture conditions for 24 hours, after which media was removed and replaced with starvation media (containing no serum or added amino acids such as L-glutamine). Autophagy inhibitors were then added to the media and the cells incubated for a further 24 hours. After this, cells were fixed in ice-cold methanol and LC3-II immunofluorescence carried out as described elsewhere (see section 2.12).

2.8 Inhibition of Autophagy in Spheroids

HT29 spheroids were grown for 15 days until they reached approximately 1mm in diameter. Spheroids were individually picked and placed in 6 well plates base coated with% (w/v) agar and incubated in 2ml of RPMI 1640 for 24 hours with Bafilomycin A1 at a concentration of 10nM. Spheroids were then collected, fixed, processed and embedded as described below.

2.9 Histology

Fixation: Spheroids were collected from spinner flasks and transferred to a universal tube where any remaining media was removed and replaced with Bouin's Solution (Sigma Aldrich, St. Louis, MO, USA). The spheroids were left in Bouin's Solution for 75 minutes before being washed in 70% ethanol to remove excess fixative and then left in 70% ethanol at room temperature until processing.

Processing: The 70% ethanol was removed, replaced with 90% ethanol for 1 hour at room temperature, which was removed and replaced with 100% ethanol for 30 minutes. This was then replaced with fresh ethanol for another 30 minutes and then repeated once more. The ethanol was removed and replaced with xylene for 30 minutes before replacing with fresh xylene and repeated one final time. Spheroids were removed from the universal tube and placed in an embedding mould, any excess xylene removed and the mould filled with liquid wax. The spheroids were left for 30 minutes in a warming oven at 68^{o}C in the wax before the waste wax was pipetted off and replaced with fresh and returned to the warming oven. This was repeated twice before the mould was placed on a cold stage to set.

Sectioning: Blocks were chilled by storing at -20^{o}C overnight. Using a microtome, 5.0µm sections of paraffin embedded spheroid blocks were cut and mounted on Superfrost Plus slides (BDH, Poole, UK). Slides were incubated on a heated stage at 37^{o}C for two hours to dry to ensure sections were fully adhered to the slide and reduce the risk of the sections coming away from the slide during subsequent use.

2.10 Haematoxylin and Eosin Staining

Sections were de-paraffinised with xylene (3x5 minutes) and rehydrated using ethanol (3x2 minutes) before being washed in running tap water for 5 minutes. Sections were

then stained with Mayer's haematoxylin (Sigma Aldrich, St. Louis, MO, USA) for 10 minutes. Excess stain was removed from the section by soaking in acid alcohol for 60 seconds before rinsing in running tap water for 5 minutes and then immersing in Scott's Tap Water for 60 seconds to allow the colour to develop. Sections were counterstained in Eosin for 60 seconds before a final wash in running tap water. Finally the sections were dehydrated using sequential ethanol (1x2minutes, 2x1minute) and xylene (3x1minute) series and mounted using diphenylxylene (BDH, Pool, UK).

2.11 Immunohistochemistry

Sections were de-paraffinised with xylene (3x5 minutes) and rehydrated using ethanol (3x2 minutes) before being washed in running tap water for 5 minutes. If necessary, antigen retrieval was then carried out by heating slides in antigen retrieval solution (see Table 1) in a microwave for a specific length of time before cooling. Slides were rinsed in tris-buffered saline (TBS) before endogenous peroxidise activity was blocked for 10 minutes at room temperature by incubating with hydrogen peroxidase block. Following this, further blocking steps were carried out depending on the primary antibody used (see Table 1). Non-specific binding was blocked by incubating the sections with Antibody Diluent containing Bovine Serum Albumin (Invitrogen, Camarillo, CA, USA) immediately prior to the application of the primary antibody. The sections were incubated with 100µl of the primary antibody diluted in Antibody Diluent in a humidified chamber. After incubation, slides were washed in TBS containing 10% v/v Tween-20 (TBST) (2x5 minutes) and TBS (1x5 minutes) before the application of 2 drops of labelled polymer-HRP secondary antibody, and then incubated in a humidified chamber at room temperature for 1 hour. Following this, slides were again washed in TBST (2x5 minutes) and TBS (1x5 minutes). Sections were then incubated with 3,3-

diaminobenzidine (DAB, DAKO North America Inc, CA, USA) for 10 minutes at room temperature, where the horseradish peroxidise breaks down the DAB via an oxidation reaction to produce a brown precipitate at the location of the antigen. Sections were washed in running tap water for 5 minutes before being counterstained using Mayer's haematoxylin (Sigma Aldrich, St. Louis, MO, USA) for 30 seconds, rinsed in tap water for 60 seconds, Scott's tap water for a further 60 seconds and finally tap water again for 60 seconds. Sections were then dehydrated using sequential ethanol (1x2mins, 2x1min) and xylene (3x1min) series and finally mounted using diphenylxylene (BDH, Dorset). A list of all antibodies used and specific conditions of IHC are presented in table 1.

Table 1. List of all antibodies used for immunohistochemistry and the specific conditions used for antibody incubations, antigen retrieval and blocking procedures.

Primary Antibody	Dilution	Incubation	Antigen Retrieval	Blocking Reagents	Secondary Antibody
Anti-LC3B antibody (APG8B, rabbit polyclonal anti-human LC3B Ig; Abgent, San Diego, CA, USA; 0.25mg/ml)	1:100	4°C Overnight	None	5min Envision H_2O_2, 30min Zymed Antibody Diluent	Labelled polymer-HRP anti-rabbit (K4010, EnVision Kit, DAKO)
Anti-Ki-67 antibody (B56, mouse anti human Ki-67 IgG_1,κ; BD Pharmingen, NJ, USA; 250μg/ml)	1:100	4°C Overnight	10 min Citrate Buffer (10mM, pH 6.0) 20 min cooling	3% H_2O_2 (v/v in methanol) Normal Horse Serum (1:200 in PBS for 20 min)	Labelled polymer-HRP anti-mouse (K4007, EnVision Kit, DAKO)
Anti-Cleaved Caspase-3 antibody (Asp175, rabbit monoclonal anti-human Cleaved Caspase-3 IgG; New England BioLabs, MA, USA)	1:800	4°C Overnight	10 min Citrate Buffer (10mM, pH 6.0) 20 min cooling	5min Envision H_2O_2, 2 hour 4% (v/v in TBS) Normal Goat Serum	Labelled polymer-HRP anti-rabbit (K4010, EnVision Kit, DAKO)
Anti-Beclin 1 antibody (BECN1, rabbit polyclonal anti-human Ki-67; Novus Biologicals, CO,USA; 0.2mg/ml)	1:400	4°C Overnight	None	60min Zymed Antibody Diluent	Labelled polymer-HRP anti-rabbit (K4010, EnVision Kit, DAKO)
Anti-H2AX antibody (20E3, rabbit monoclonal anti Phospho-Histone H2A.X IgG; Cell Signalling, MA, USA)	1:400	1 hour RT	10 min Citrate Buffer (10mM, pH 6.0) 20 min cooling	3% H_2O_2 block, 10min Zymed Antibody Diluent	Labelled polymer-HRP anti-rabbit (K4006, EnVision Kit, DAKO)

2.12 Immunofluorescence

HT-29 and DLD-1 cells (2 x 10^4) were seeded onto 2cm^2 coverslips and placed into 6 well plates with 2ml of RPMI 1640 (ATCC, Manassas, VA, USA) media containing 10% FBS for 24 hours. Media was subsequently removed and replaced with 2ml of media containing no serum for a further 24 hours. The starvation media was then removed and the coverslips were incubated on ice for 5 minutes with 2ml of ice cold methanol to fix the cells. The methanol was removed and non-specific binding was blocked by incubating coverslips with 2ml of 2% (w/v) BSA (Bovine Serum Albumin, Sigma Aldrich, St Louis, MO, USA) in PBS (Phosphate Buffered Saline, Sigma Aldrich, St Louis, MO, USA) for 60 minutes at room temperature. Coverslips were washed for 2 x 5 minutes in 2 ml of PBS before incubating for 45 minutes at room temperature with 200µl of rabbit anti human LC3-II primary antibody (APG8A, rabbit polyclonal anti human LC3B; Abgent, San Diego, CA, USA; 0.25 mg/ml) diluted 1:50 with 2% BSA in PBS. Coverslips were then washed 3 x 5 minutes with PBS before application 200µl of the secondary fluorescent antibody (Alexa Fluor® 546, goat anti-rabbit, IgG; Invitrogen, Camarillo, CA, USA, 2mg/mL) diluted 1:50 in 2% BSA in PBS. Coverslips were incubated at room temperature for 45 minutes before washing for 3 x 5 minutes with PBS and then mounted using VECTORSHIELD hard-set mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) and kept in the dark at 4^{o}C until examination under the microscope.

2.13 Preparative Fluorescence Associated Cell Sorting (FACS)

Upon growing to an average diameter of 1500µm, HT29 spheroids were incubated with 1µM Hoechst 33342 (Invitrogen, Camarillo, CA, USA) for 30mins at 37^{o}C before gently washing in medium. Spheroids were then incubated in 0.25% (w/v) trypsin for

15mins at 37^{o}C before transferring to medium and vigorously pipetting until a single cell suspension was achieved.

The cells were sorted into different populations (necrotic core and viable rim see Figure 1) based on their cell size (FSC) and granularity (SSC). These two populations were then further sorted by the intensity of Hoechst staining using an excitation wavelength of 350-360nm and emission monitored at 488nm. Each population was split into 3-4 different groups with roughly 25-30% of the population in each. A total of 7 fractions were collected, 3 derived from the necrotic core population and 4 from the viable rim population. The fractions were collected on ice in McCoy's 5A Modified Medium with 20% FBS and 10% Penicillin Streptomycin (Sigma Aldrich, St Louis, MO, USA) .

Acknowledgements go to Dr Andrei Mardaryev for his knowledge and expertise in this experiment.

2.14 Analysis of Fractions

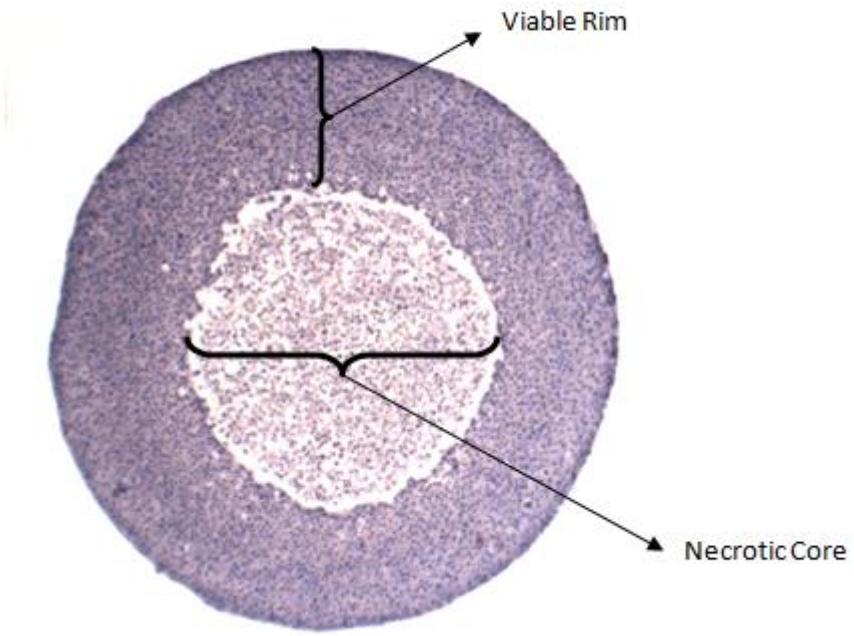


Figure 1. A HT-29 MCTS Haematoxylin and Eosin stained section showing the location of the viable rim and necrotic core.

2.14.1 Plating Efficiency

To determine the viability of the cells from each fraction the plating efficiency (PE) was determined. A number of concentrations (1000, 100, 50 and 10 cells/ml) of cells were plated onto six-well plates with 3mls medium and incubated at 37^{o}C, 5% CO_2, 95% humidity. After 10 days colonies containing more than 50 cells were counted, and PE was determined using the following formula

PE (%) = (Number of colonies formed /Number of cells plated) x 100

2.15 Formation of Necrotic Core Clones

Cells from the least fluorescent (hence most central to the spheroid) necrotic core fraction were plated onto six-well plates at various low concentrations (10-100 cells/ml) and allowed to grow for 2-3 weeks. In wells where only a single colony had formed it was transferred into a T25 cell culture flask with fresh media and allowed to grow further. Cell lines derived from these clones were given the notation NCC to denote necrotic core clone. Control clones were also established from the parental cell line using the same method only with more dilute cell concentration (1-20 cells/ml). These were given the notation CC.

2.16 Necrotic Core Clone Growth Curves

Monolayer cultures in the exponential phase of growth were washed with Hanks Balanced Salt Solution, and incubated with 0.25% Tryspin for 5-15 minutes until a single cell population was achieved. Once the cells were detached, the trypsin was diluted with 10ml medium and the cells collected and centrifuged at 1000*g* for 5 minutes. Cell pellets were then re-suspended in medium, and a cell count determined using a haemocytometer. Cells (10^3) were then added to 15 T25 flasks, and left to incubate at 37^{o}C in 95% humidity, 5% CO_2. On Day 1, 2, 3, 4 and 7, the above process

was repeated in order to get an average cell count for that day. Doubling Times were established (determined) from the log phase of the growth curves.

3 Results

The findings of this chapter of work will be presented in the next section detailing the isolation of living cells from within the necrotic core of MCTS and their characterisation .

3.1 HT-29 and DLD-1 multicellular tumour spheroid growth curves.

HT-29 cell line readily formed spheroids when single cell suspensions were added directly to medium in spinner flasks and constantly stirred at a rate of 50rpm. There was no need to use standard liquid overlay techniques to 'seed' spheroids and this also removed the need to select spheroids of a defined size prior to transfer to spinner flasks. Small spheroids were visible to the naked eye after 72 hours and spheroid diameter was determined using an inverted microscope fitted with a calibrated eyepiece graticule. The DLD-1 cell line required 24-72 hour liquid overlay incubation prior to transfer to the spinner flask to induce the MCTS to form. The growth of DLD1 and HT-29 spheroids is presented in figure 1. DLD1 spheroids reached a maximum diameter of 800-1000µm after 14-17 days, HT-29 spheroids reached an average size of 1500µm after 21 days, with individual spheroids reaching a maximum diameter of in some cases 2000µm (see Figure 2). The growth of the spheroids was biphasic, in the first 6 days of growth the spheroids grew exponentially, doubling in size every 31.4 hours (HT-29) and 40.2 hours (DLD-1), after this time period the growth slowed down to give a doubling time of 201.7 hours (HT-29) and 189.6 hours (DLD-1). The first phase of MCTS growth coincides with the period of growth prior to development of the necrotic core. The second slower phase of growth begins around the time of necrotic core formation (~500µm). Continued culture after both cell lines have reached their maximal sizes

resulted in the loss of structure and eventually breakdown of the spheroids even in the presence of fresh, nutrient rich medium.

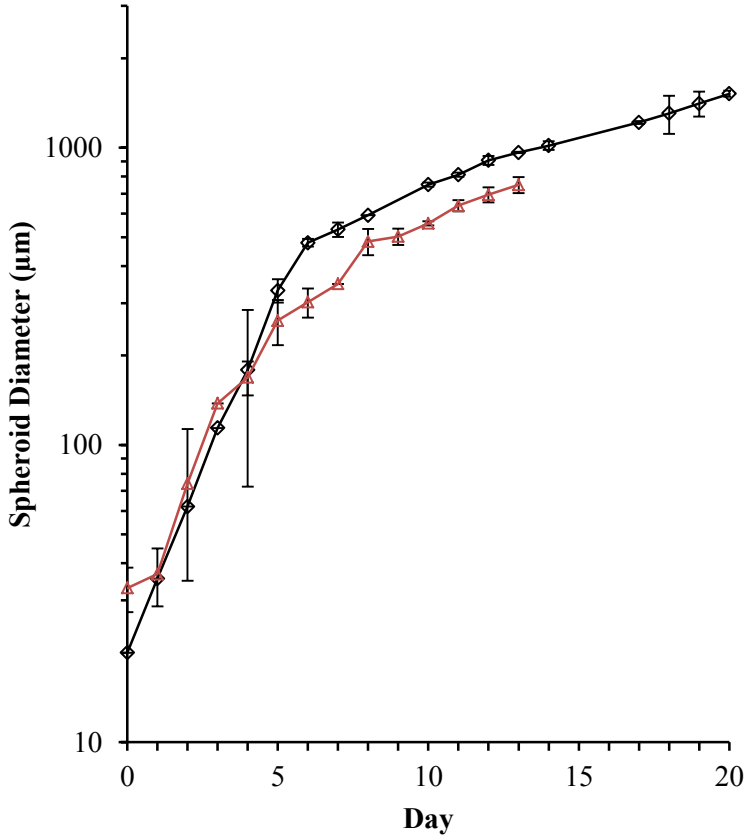


Figure 2. Graph showing the respective growth of HT-29 spheroids (shown in black) and DLD-1 spheroids (shown in red) grown in spinner flasks where the medium is changed as required. Three separate experiments were carried out in order to calculate an average and standard deviations for the spheroid growth. Cells were transferred to the flask on Day 0.

3.2 Growth of HCT116 as MCTS.

HCT-116 cells were unable to form MCTS directly from single cell suspensions. Culturing the cells using liquid overlay techniques (1% agar in T-75 flasks) for 24-72 hours also failed to encourage them to form MCTS prior to transfer to a spinner flask. When introduced to culture conditions in the spinner flasks, the cells stopped growing. The method which was found to be successful for this cell line involved seeding a number of cells (7.5×10^3 in 200µl medium) into agar coated (1%) wells of a 96 well

plate and incubating them for several days before transfer to a spinner flask. The result of this was the formation of one MCTS per well. At the time of transfer to spinner flasks, MCTS can be as big as 600-700µm in diameter. Once transferred to the spinner flasks, HCT-116 MCTS will continue to grow until a maximal diameter of approximately 1000µm is reached. This final growth phase take 5 days upon transfer to spinner flask.

3.3 Morphology of MCTS

All three cell lines formed spheroids that had a similar morphology characterised by the presence of a viable rim and a centralised necrotic core.

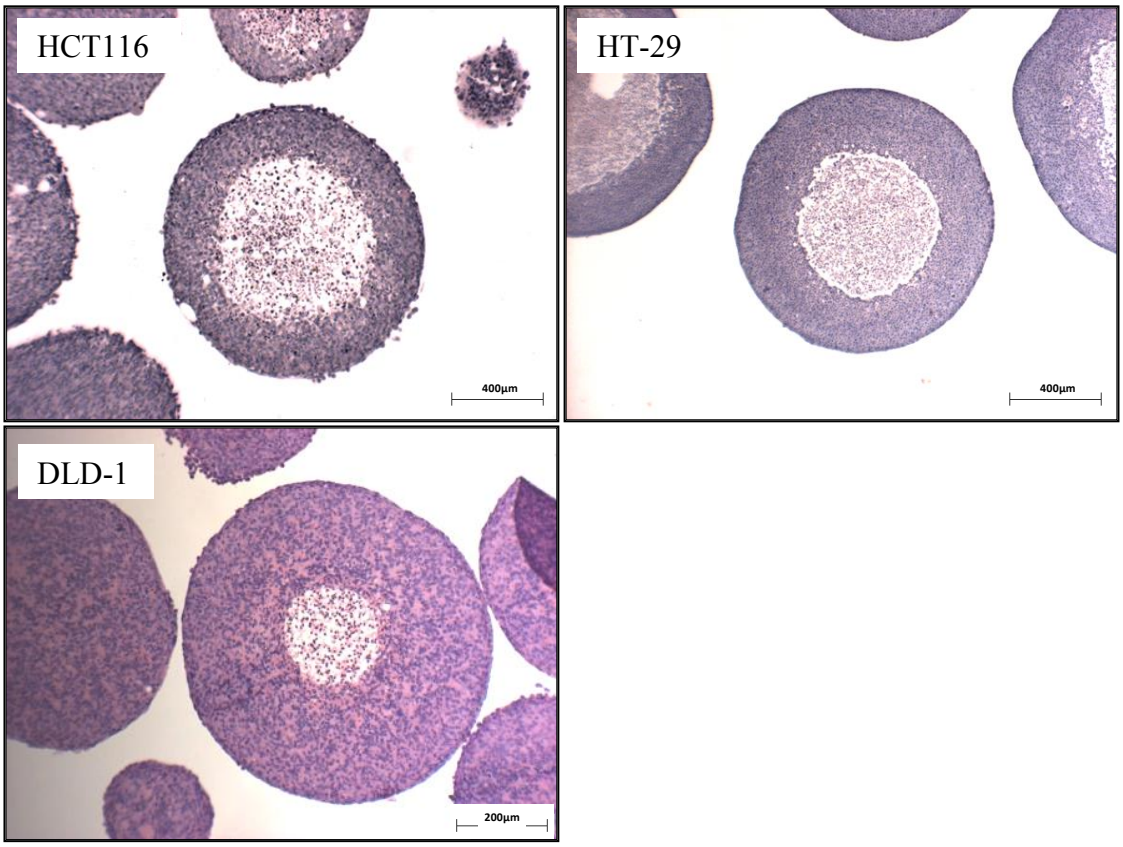


Figure 3. Cross sections through HCT116, HT-29 and DLD-1 MCTS stained with Haematoxylin and Eosin.

HT-29, HCT-116 and DLD-1 cell lines all formed large spheroids, particularly HT-29s which grew in some cases to over 2000µm in diameter. As the spheroids grew a necrotic core formed (Figure 3) when the spheroid reached approximately 500µm in

diameter. The formation of a necrotic core occurs when the diffusion distance from the outer layer of cells to the innermost cells is too far for sufficient nutrients and oxygen to penetrate. As the spheroid is observed to grow larger in diameter the necrotic core itself expands as more cells are too far from the surface layer to survive. DLD-1 spheroids were harvested at various different time points within a 15 day period then fixed, embedded, sectioned and stained with haematoxylin and eosin in order to examine how the morphology of MCTS changes are they grow larger. The results are presented in Figure 4.

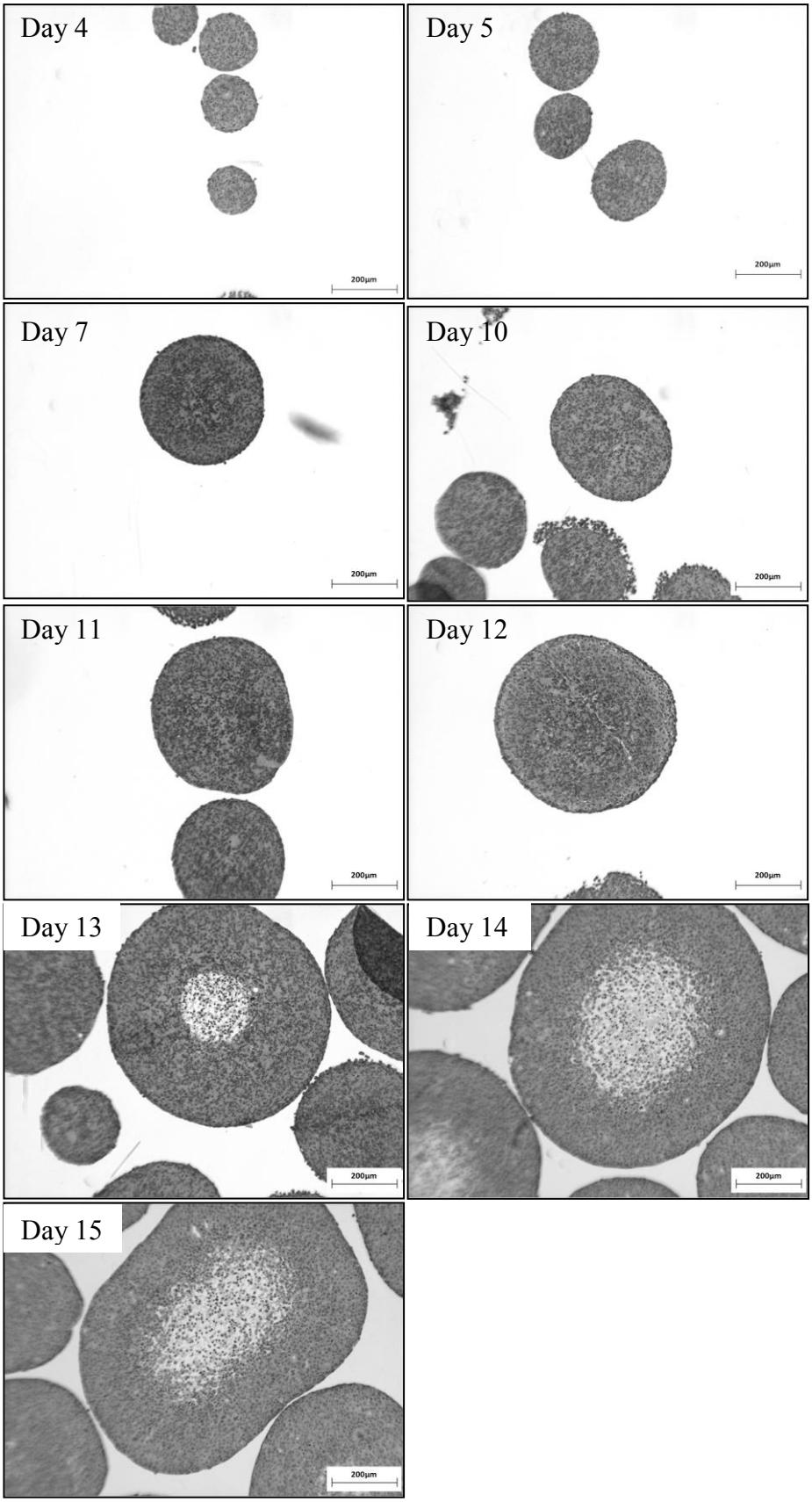


Figure 4. DLD1 MCTS sections stained with haematoxylin and eosin, depicting the growth curve of DLD1 spheroids. Scale bar represents 200µm.

Measurements of the diameter of the necrotic core and the viable rim as a function of overall spheroid diameter are presented in Figure 5. As the spheroid grows, the necrotic core increases in size but the width of the viable rim tends to stay a relatively constant thickness after the formation of the necrotic core, ranging from between 100-250µm.

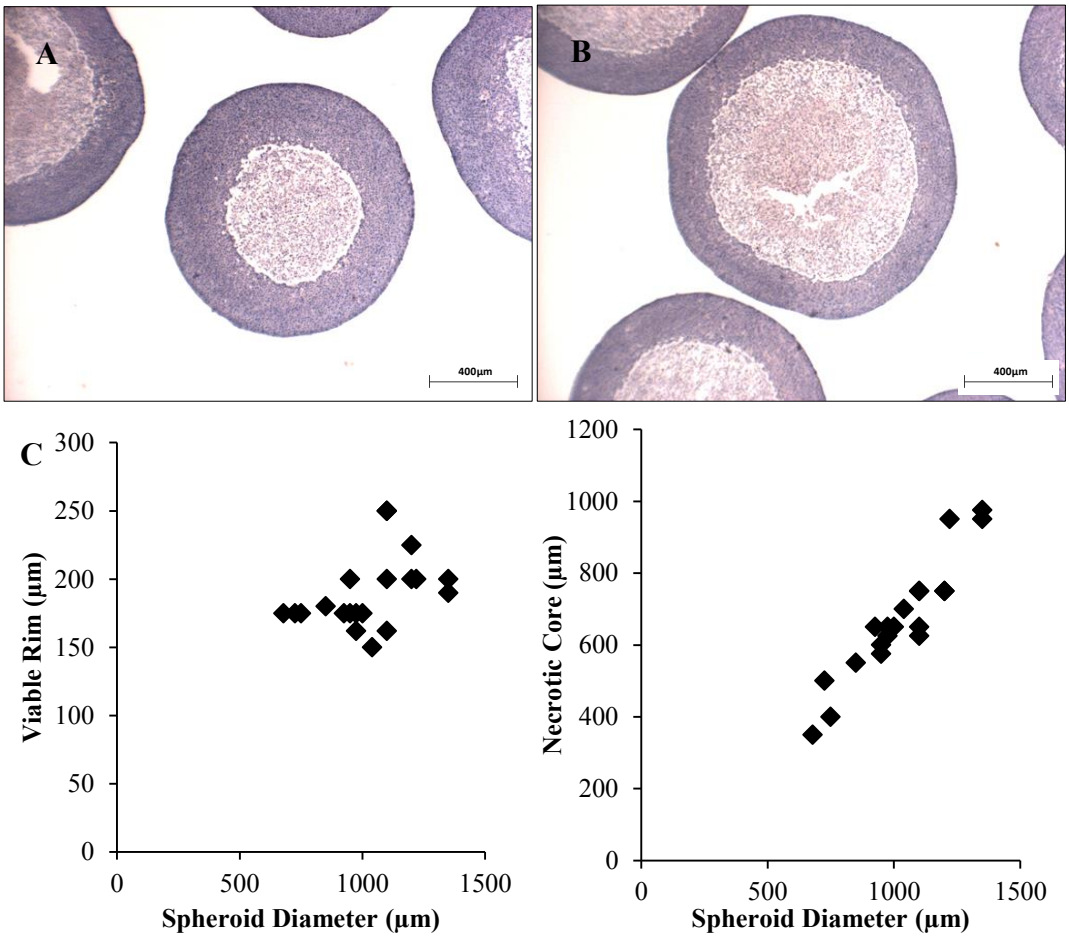


Figure 5. Spheroid Diameter and the relative size of the necrotic core and viable rim. Panel A and B show Haematoxylin and Eosin staining of two HT-29 spheroid sections, one showing a larger spheroid with a thinner viable rim and larger necrotic core (A), and one smaller spheroid with a comparatively thicker viable rim and smaller necrotic core (B). Panel C presents the relationship between spheroid diameter and the size of the viable rim and necrotic core in HT-29 MCTS. For each MCTS 4 readings were taken for VR and NC thickness and used to determine an average. In total 20 MCTS were examined per group with serial sections examined to ensure the widest part of the MCTS is being examined. Experimental errors were omitted from the graph in the interest of clarity.

As the spheroid increases in size, the diameter of the necrotic core also increases (Figure 5 and Figure 6). The graphs show that as the size of the spheroid increases the

thickness of the viable rim stays relatively constant, in comparison to the necrotic core which increases in size. This is seen in both HT-29 (Figure 5) and DLD-1 (Figure 6). The implication being that the diffusion distance stays relatively constant and is the driving factor behind the formation and size of the necrotic core. The Pearson's product-moment correlation coefficient (P) was calculated to determine the straight line correlation between the size of the spheroid and the necrotic core size. Where 0 = no correlation, -1 = negative correlation and +1= positive correlation, the results demonstrate that there is a positively correlated relationship between spheroid diameter and size of the necrotic core for both DLD-1 (P = 0.927) and HT-29 (P = 0.942) spheroids.

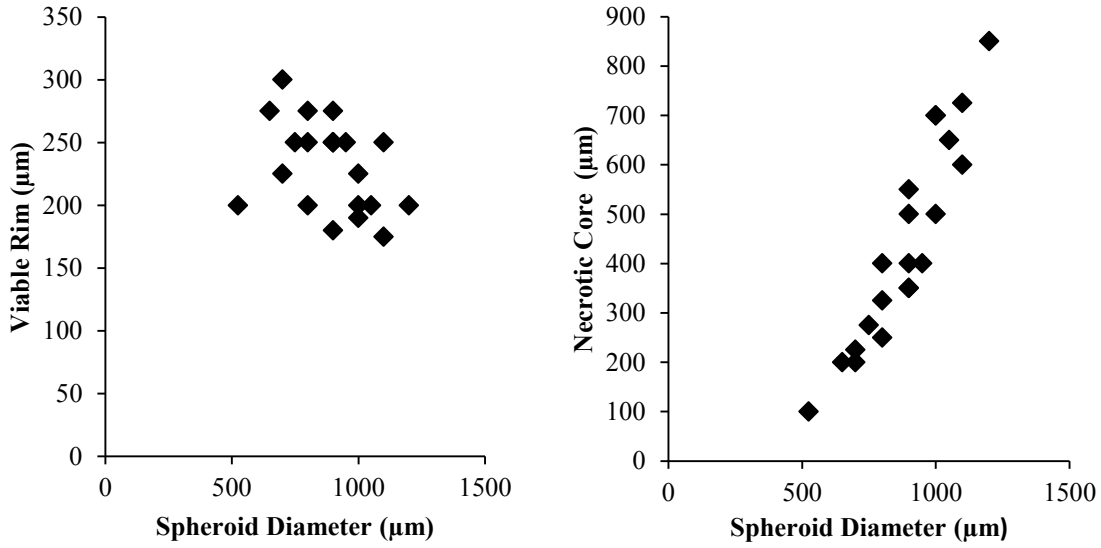


Figure 6. Data demonstrating the relationship between spheroid diameter and the relative sizes of the viable rim and necrotic core in DLD-1 MCTS. Panel A and B show data demonstrating the relationship between spheroid diameter and the relative size of the viable rim and necrotic core in DLD-1 MCTS. Panel A showing the necrotic core as a function of MCTS size, panel B showing the viable rim as a function of MCTS size.

3.4 Ki67 staining highlighting cellular proliferation within MCTS.

HT-29 and HCT116 MCTS sections were stained with an antibody against Ki-67, a marker of cellular proliferation (Figure 7). In HT-29 spheroids, only cells in the outer half of the viable rim stained positive for Ki67. The subset of cells within the viable rim which were proliferating in the HT-29 MCTS were located in the outer 100µm of the spheroid rim, whilst cells localised any deeper than this were quiescent (Figure 7A). In a large HT-29 spheroid (>500µm) with a well-defined necrotic core, there are no cells actively proliferating within the necrotic core. In the smaller HCT-116 MCTS the majority of actively proliferating cells were seen in the outer 100µm of the spheroid viable rim however a number of proliferating cells were seen in the inner half of the viable rim (Figure 7B). The 100µm limit which seems to encompass the majority of proliferation cells in both types of MCTS would suggest that 100µm is a crucial diffusion distance for nutrients and/or oxygen to support cell replication in both HT-29 and DLD-1 MCTS.

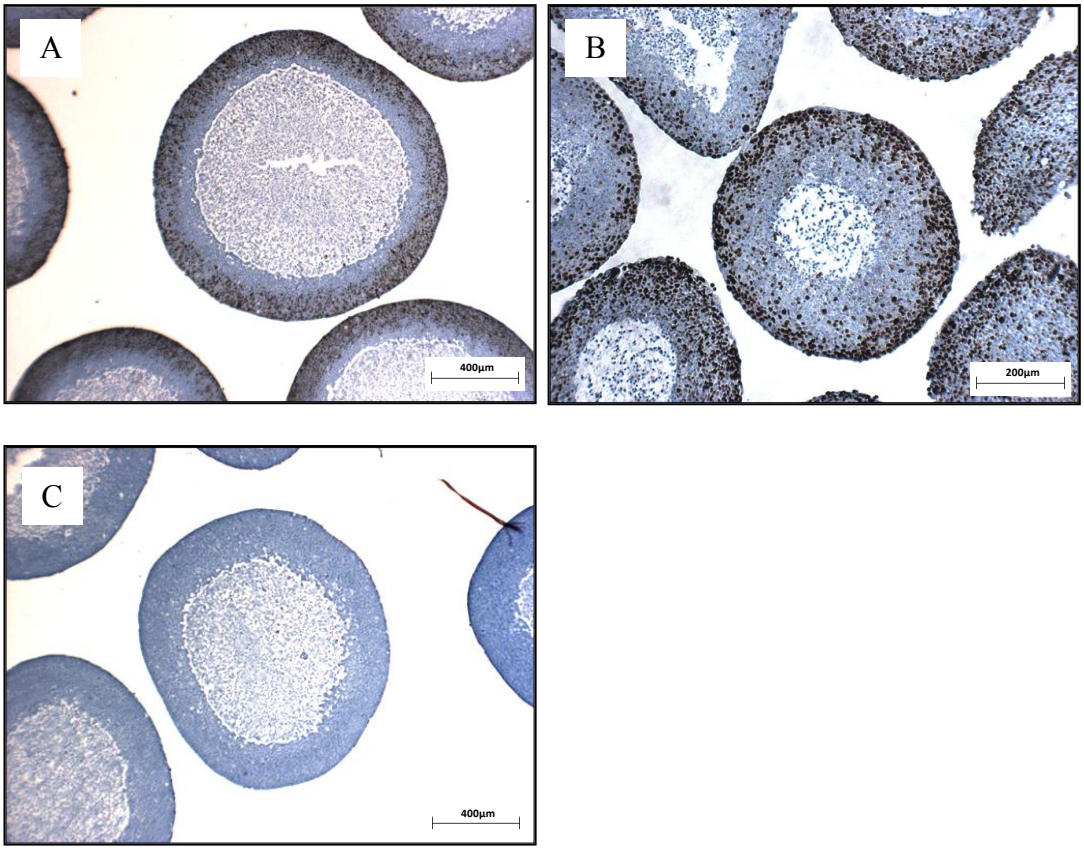


Figure 7. Cellular proliferation within MCTS. Panel A presents the expression of Ki-67 in a HT-29 MCTS. Panel B showing Ki-67 expression in a HCT 116 MCTS. Panel C the negative control, a HT-29 spheroid incubated without primary antibody.

3.5 Pore formation in the viable rim of large spheroids.

As described previously, the viable rim becomes very thin in relation to the necrotic core as the spheroid increases in size. When spheroids become large, pores can form in the viable rim as illustrated in Figure 8. The characteristic feature of pore formation is that whilst the majority of the viable rim remains intact, a focal thinning of the viable rim occurs in places and when complete, the necrotic core can be extruded (Figure 8). This process mostly occurs in spheroids when they reach a size of between 1500-2000µm. These events become more visible when large mature MCTS are removed from the spinner flasks and placed in static liquid overlay conditions. Under continuous stirring conditions in the spinner flasks, MCTS appear to retain their compact structure

but when placed in liquid overlay conditions, pore formation becomes more visible and the spheroid becomes more fragile. It is not known whether the transfer to liquid overlay accelerates the formation of pores or it reflects the fact that constant stirring will remove any extruded material making the pores harder to detect visually. This phenomenon is more common in the HT-29 cell line and pore formation was not common in DLD-1 spheroids. Whether this is just an innate characteristic of this particular cell line or caused by its ability to grow to the large sizes needed for the process to occur is unclear. A HT-29 MCTS demonstrating the formation of a pore in the viable rim and subsequent release of the inner necrotic core cells was also stained for Ki-67. The staining shows the cells 'escaping' from within the MCTS are not the actively proliferating Ki-67 positive cells from the viable rim but those from the necrotic core which have a stronger need to escape the nutrient poor environment in the centre of the MCTS.

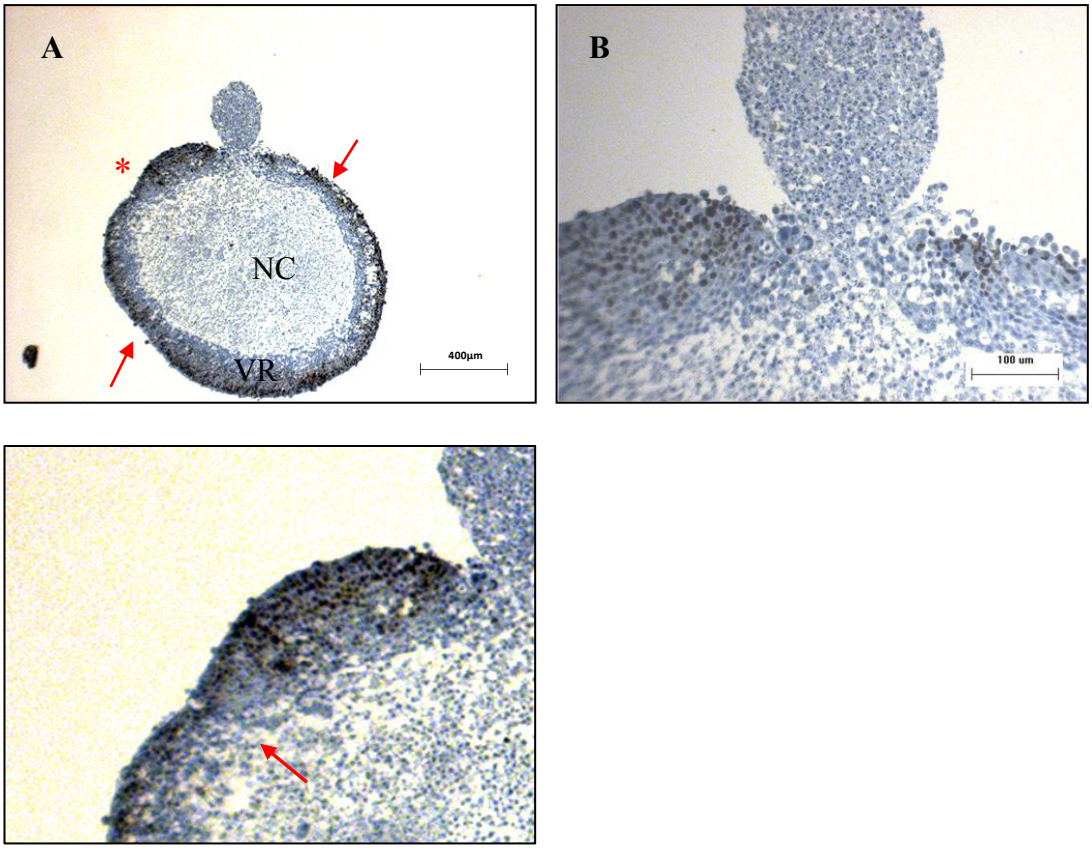


Figure 8. Cross section through HT-29 MCTS focusing on the viable rim and pore formation. Panels A and B show different magnifications of a single HT-29 spheroid stained with antibodies to Ki-67 with a pore in the viable rim. In panel A NC denotes the necrotic core and VR the viable rim of the MCTS and arrows indicate thinning of the viable rim in additional places in the MCTS. Panel C shows a close up of a section of Panel A denoted by an asterix, where the early stages of pore formation in the viable rim are visible, the thinking of the viable rim is shown by an arrow. Spheroids were cultured in liquid overlay following culture in spinner flasks and extrusion of the necrotic core is clearly visible as Ki-67 negative cells.

3.6 Detection of cells undergoing apoptosis using cleaved caspase 3 staining

MCTS sections were stained with antibodies against Cleaved Caspase-3, an effector protein involved in both the intrinsic and extrinsic apoptotic pathways, to look for the distribution of apoptotic cells within different regions of the MCTS. Within the viable rim of spheroids, only a few cleaved caspase-3 positive cells were visible with the majority of these being found close to the necrotic core boundary (Figure 9A). As expected, positively stained cells were abundant within the necrotic core (Figure 9A).

At higher magnification however (Figure 9B), a number of negatively stained cells were visible. Morphologically, these cells resembled viable cells in that they had a defined nucleus that was not fragmented. This suggests that the necrotic core is not made up just of dead and dying cells as previously thought but a number of cells have managed to adapt and survive in this harsh environment. Either that or the cells are undergoing a cleaved caspase-3 independent form of cell death. Viable cleaved caspase 3 negative cells were not just seen on the periphery of the necrotic core which would suggest that they had recently detached from the hypoxic fraction but were present throughout the necrotic core. Viable cells were even in the very centre at depths as deep as 600µm from the surface.

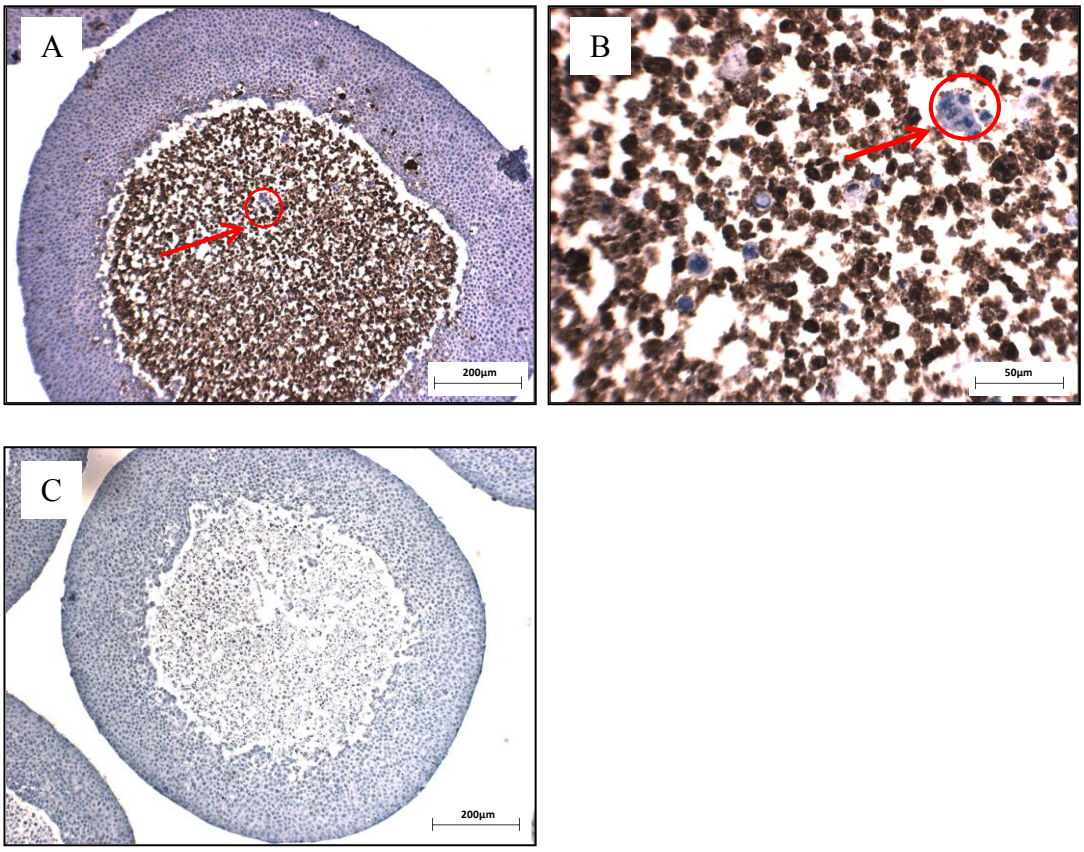


Figure 9. Immunohistochemical images showing Cleaved Caspase-3 expression in HT29 MCTS. Panel A shows the expression in the entire MCTS. Panel B shows the expression within the necrotic core. Circled in panel B are a cluster of negatively stained cells in the necrotic core. Panel C represents HT-29 spheroids stained with secondary antibody only (no primary antibody).

3.7 Analysis of necrosis in colorectal cancer xenografts

Xenografts from all three cell lines were also examined histologically to determine whether the areas of necrosis seen in MCTS were also seen when the cells were grown *in vivo*, and thus determine how representative MCTS are as a model for studying necrosis *in vitro*. Extensive areas of necrosis were seen in all three xenografts surrounded by areas of more viable tissue.

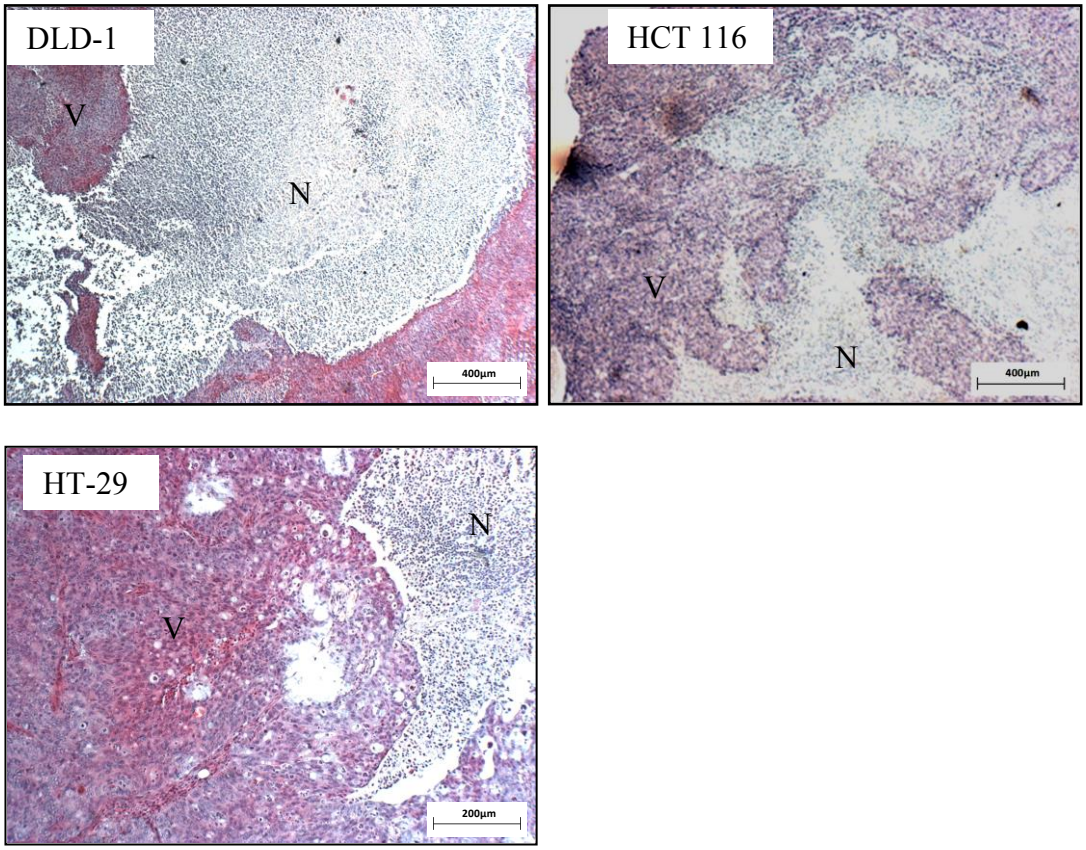


Figure 10. DLD-1, HCT-116 and HT-29 xenografts showing evidence of areas of necrosis. Necrotic areas are indicated with an N, areas of viable tissue are indicated with a V.

Once areas of necrotic tissue were seen in haematoxylin and eosin stained HT-29, DLD-1 and HCT 116 xenografts (Figure 10), these same tissues were also examined immunohistochemically to determine their cleaved caspase-3 expression (Figure 11). The necrotic regions of multicellular tumour spheroids are symmetrical unlike the areas of necrosis seen in the same cells grown as *in vivo* xenografts. In all three xenografts expression of cleaved caspase-3 was seen located in areas of necrotic tumour tissue.

Similar to the expression seen in HT-29 MCTS, not all the necrotic cells were positive for cleaved caspase-3. In the HCT 116 tissue only a small proportion of cells can be seen to be stained whereas in the HT-29 and DLD-1 xenografts a larger proportion can be seen to be positive.

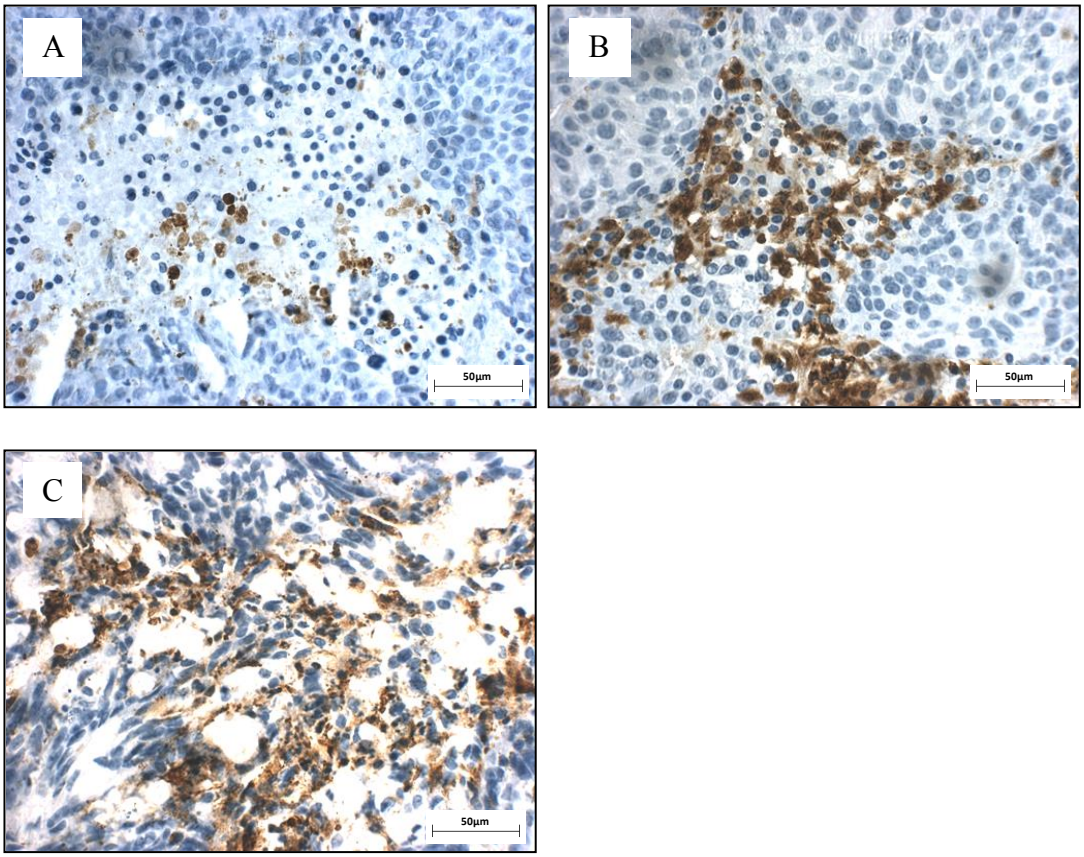


Figure 11. Immunohistochemical analysis of the expression of cleaved caspase-3 in Panel A HCT 116, Panel B DLD-1 and Panel C HT-29 xenografts.

3.8 Comparison of necrotic tissue in clinical colorectal liver metastases and HT-29 Multicellular Tumour Spheroids

In colorectal liver metastases, the morphology resembles that of MCTS with a central area of necrotic tissue surrounded by a ring of viable cells (Figure 12). The cells within the necrotic centre of the 'rosette' (Figure 12B) include those which still retain a definite nucleus and those in which the nucleus has broken down into multiple nuclear bodies. The combination of whole cells and cellular debris is mirrored in the HT-29

MCTS. Furthermore as in the MCTS the cell-cell connections in the necrotic areas look like they have in some places broken down leaving the cells to exist in a less rigid more 'liquid-like' state. The similarities seen in structure and morphology demonstrate that MCTS retain many features observed in clinical disease such as areas of necrotic tissue and this further supports the fact that MCTS provide a representative *in vitro* model for metastatic colorectal cancer.

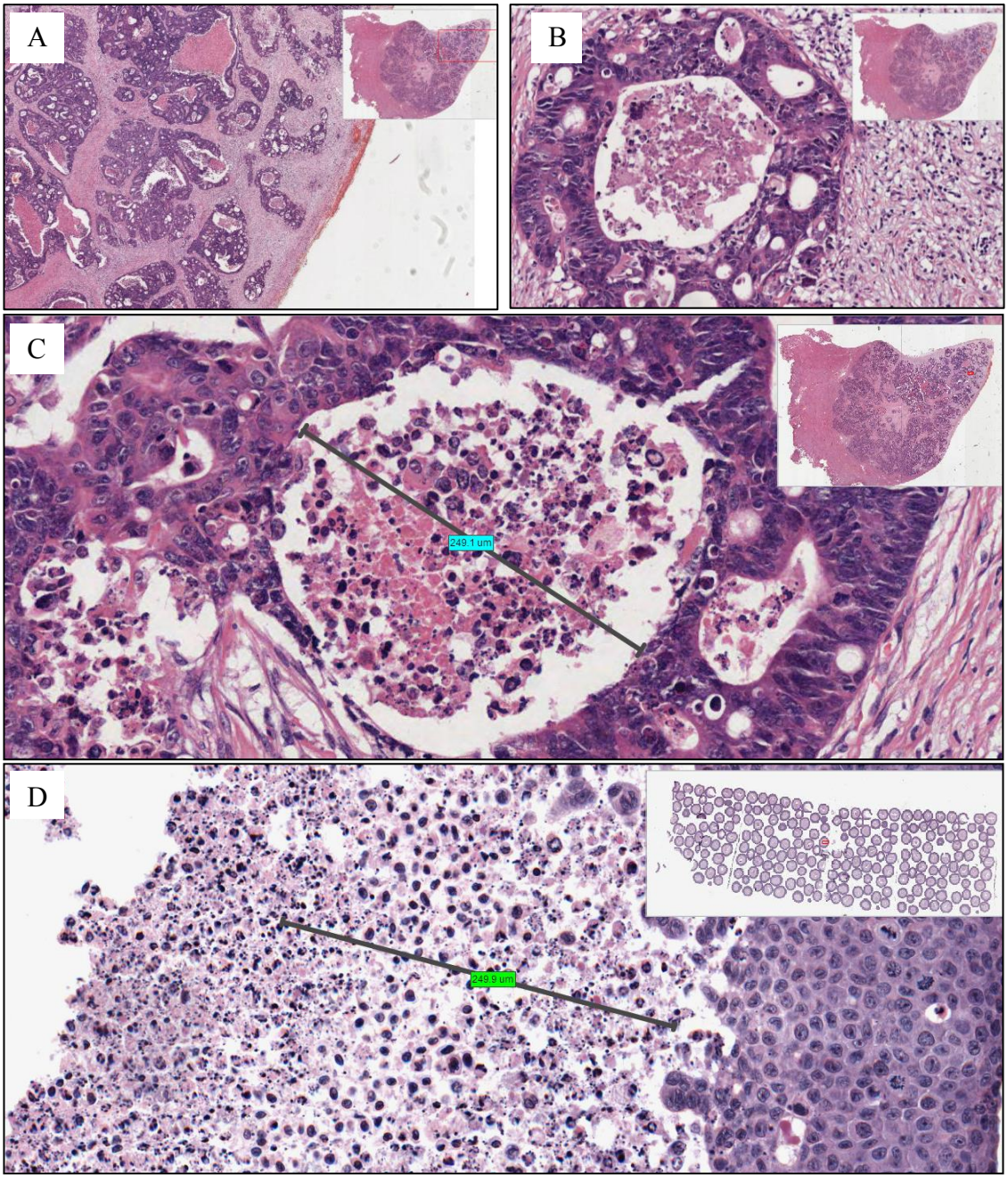


Figure 12. Morphological analysis of necrotic tissue in colorectal liver metastases and it's similarities to the necrotic core in HT-29 MCTS. Panel A shows a liver section containing colorectal metastatic deposits. Inset entire liver section, red square represents the field of view in the main image. Panel B shows the morphology of a rosette consisting of a central mass of necrotic tissue surrounded by a ring of viable cells. Inset entire liver section. Panel C shows higher magnification (x20 objective) of the necrotic centre of the rosette. Inset entire liver section, grey line indicates the diameter of the necrotic area. For comparison panel D shows the necrotic core of a HT-29 MCTS (x20 magnification). Inset number of MCTS sectioned together, grey line represents the same distance seen in the necrotic liver rosette for comparison. Images were provided by University of Leeds Institute of Cancer and Pathology, Department of Pathology, Anatomy and Tumour Biology.[3]

3.9 Autophagy in MCTS

The catabolic process of macroautophagy is known to be involved in cell survival in hostile environments. The discovery of cleaved caspase-3 negative cells within the necrotic core led to the investigation of whether alternative survival and death mechanisms were engaged, including autophagy.

3.10 Characterising Autophagy inhibitors

In order to determine the presence of autophagic flux within a cell an appropriate autophagy inhibitor must be used. The autophagy inhibitor must inhibit the process of autophagy after the formation of the (LC3-II coated) autophagasome, allowing these to build up within the cell if it is actively undergoing autophagy. If the autophagy inhibitor inhibits the process before the formation of an autophagasome then all results seen will be negative regardless of the state of autophagic flux within the cell. It was found that the addition of both bafilomycin A1 (10nM) and chloroquine (50mM) significantly increased the number of LC3 puncta, and therefore autophagasomes, in the starved DLD-1 monolayers, whilst 3-methyladenine had a somewhat reduced effect (Figure 13). Therefore both bafilomycin A1 and chloroquine are suitable inhibitors for use in measuring autophagic flux.

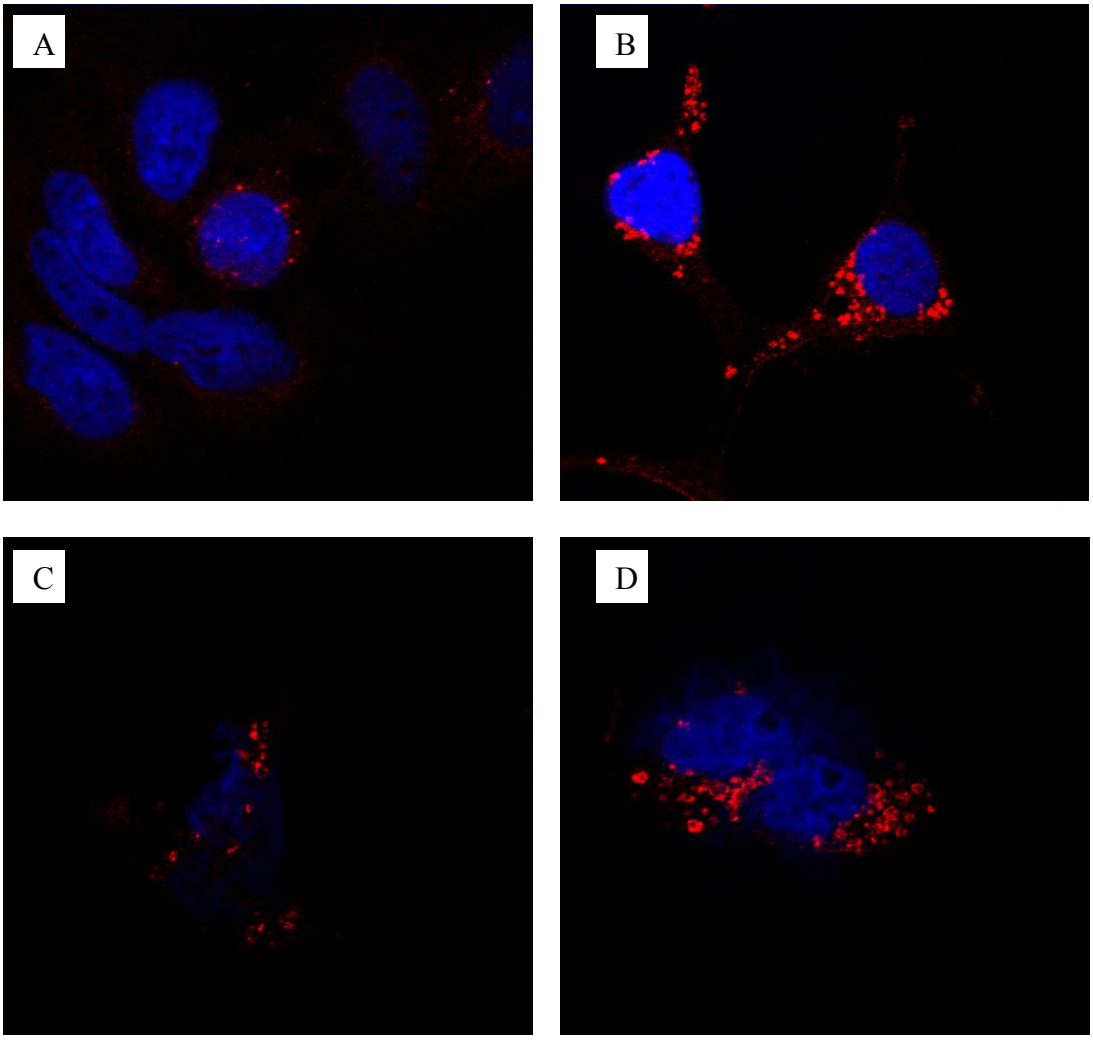


Figure 13. Immunofluorescent images showing the expression of LC3 and the effect of different autophagy inhibitors on DLD-1 cells. Red fluorescence represents LC3-II, nuclei are counterstained blue. Panel A 24hr starvation, no autophagic inhibitor. Panel B 24hr starvation and 10nM bafilomycin. Panel C 24hr starvation and 5mM 3-methylalanine. Panel D 24hr starvation and 50μM chloroquine.

3.11 LC3-11 and Beclin-1 staining in HT-29 MCTS

To detect autophagy in MCTS, various techniques were used. Spheroids which had been pre-treated prior to fixation with bafilomycin A1 (10nM) were sectioned and stained with an anti-LC3-B antibody and compared to sections of untreated spheroids to look for punctate expression of the protein within the different regions of the spheroid. Beclin-1 was also used as an autophagic marker to support the results seen from the LC3 staining.

As shown in Figure 14, LC3 was found to be expressed in punctate dots in the necrotic core (Panel C and D). The number of these puncta were also shown to qualitatively increase in the necrotic core when spheroids were treated with autophagy inhibitor Bafilomycin A1 for 24 hours (Panel D) This increase means that autophagy is actively occurring in some cells within the necrotic core, as more autophagasomes are being produced. Beclin-1 expression was also seen located in the necrotic core of the spheroid (Panel B), further validating the LC3-II staining and the conclusion that cells in the necrotic core of MCTS are actively undergoing autophagy.

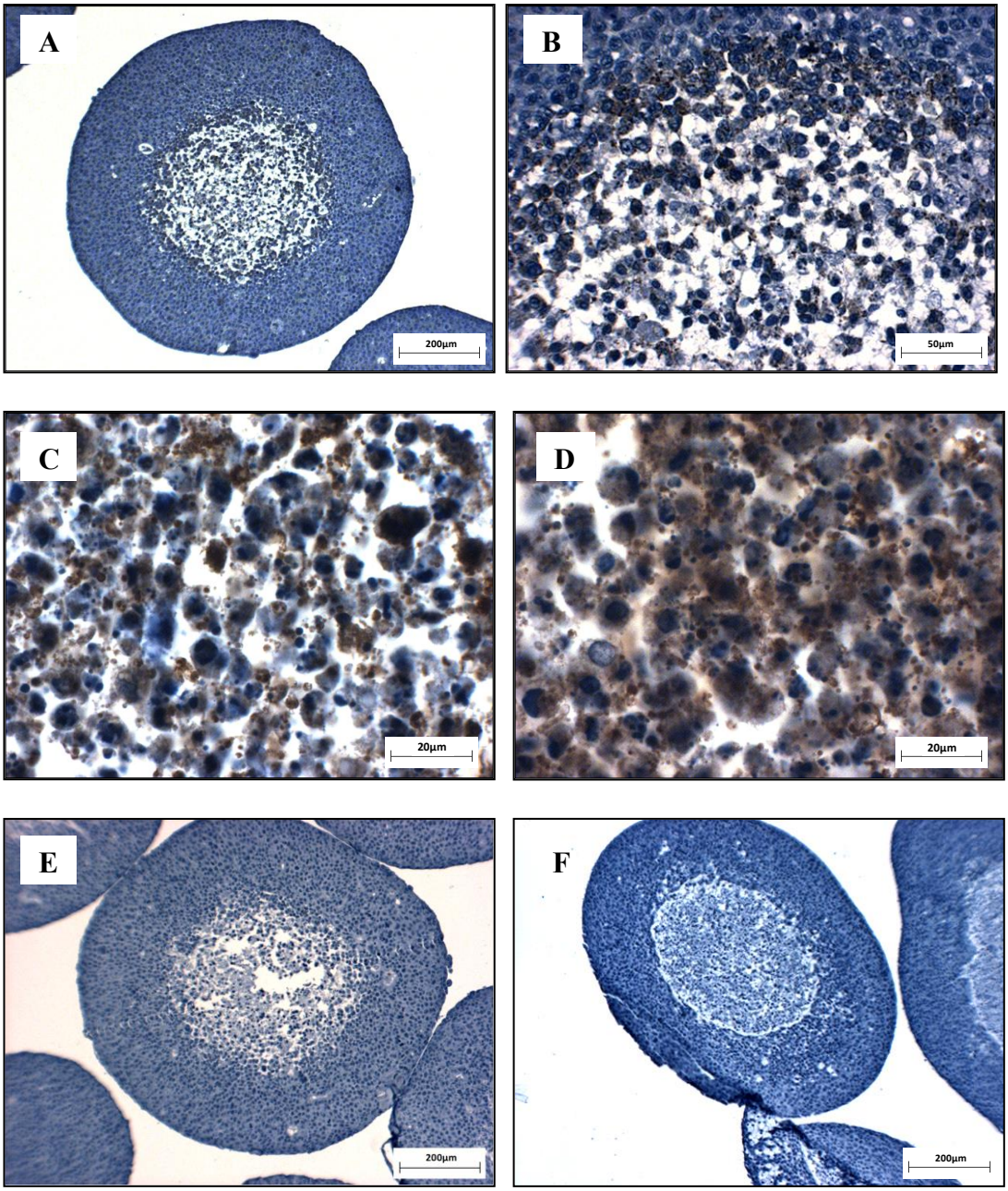


Figure 14. Immunohistochemical staining of HT29 spheroids for Beclin 1 and LC3-II. Panel A shows a whole spheroid stained with anti-Beclin-1 antibody. Panel B shows the positive Beclin-1 staining at the viable rim/necrotic core boundary and in the necrotic core. Panels C and D show the necrotic core stained with anti-LC3 antibody where panel C shows staining of an untreated spheroid and D shows the necrotic core of a spheroid that has been treated with the autophagy inhibitor Bafilomycin A1. Panels E and F are primary antibody controls (no primary antibody added) for Beclin-1 primary LC3-II respectively.

3.12 DNA double strand breaks in cells within the Necrotic Core

To detect whether the cleaved caspase-3 negative cells within the necrotic core were indeed alive and managing to survive, HT-29 MCTS sections were investigated for the expression of phosphorylated H2AX, a marker of the DNA damage response specifically to DNA double strand breaks, that can also be expressed in senescent cells. As shown in Figure 15 a number of cells on the boundary between the necrotic core and viable rim were stained positive for phorphorylated H2AX (p-H2AX). When examined closely it was found that some cells within the necrotic core were also positive. As in the case of the cleaved caspase-3 negative cells, p-H2AX positive cells were seen at similar depths within the necrotic core. It is not known whether the cleaved caspase-3 negative cells are positive for p-H2AX.

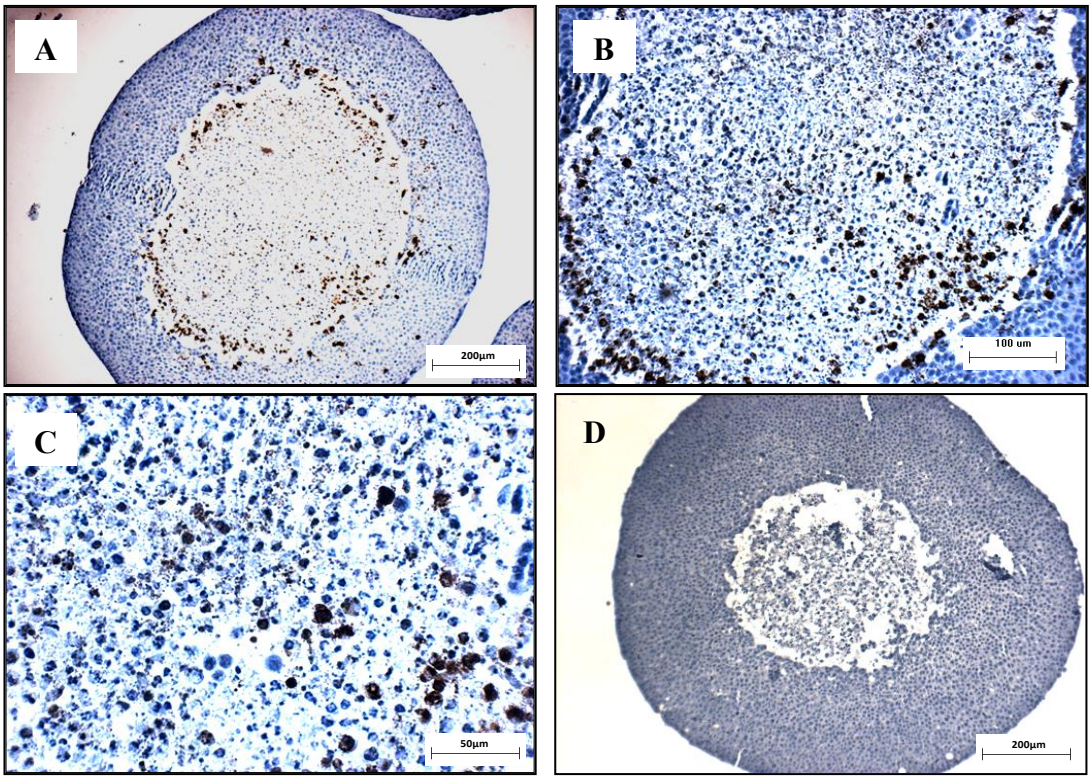


Figure 15. Images showing p-H2AX immunohistochemical staining on HT-29 MCTS sections. Panel A represents a low magnification (X10 objective) covering the entire MCTS, whereas panels B and C focus on the necrotic core using a X10 and a X20 objective lens respectively. Panel D represents the primary antibody control (no primary antibody added).

3.13 Phosphorylated-H2AX expression *in vivo.*

To see whether the same expression of H2AX could be seen *in vivo* as well as *in vitro*, sections from HT-29, DLD-1 and HCT 116 xenografts were stained with antibodies against H2AX. As seen in Figure 16 positive expression of p-H2AX was seen in all three xenografts in or adjacent to areas of necrosis. This is consistent with the pattern of p-H2AX expression observed in MCTS *in vitro*.

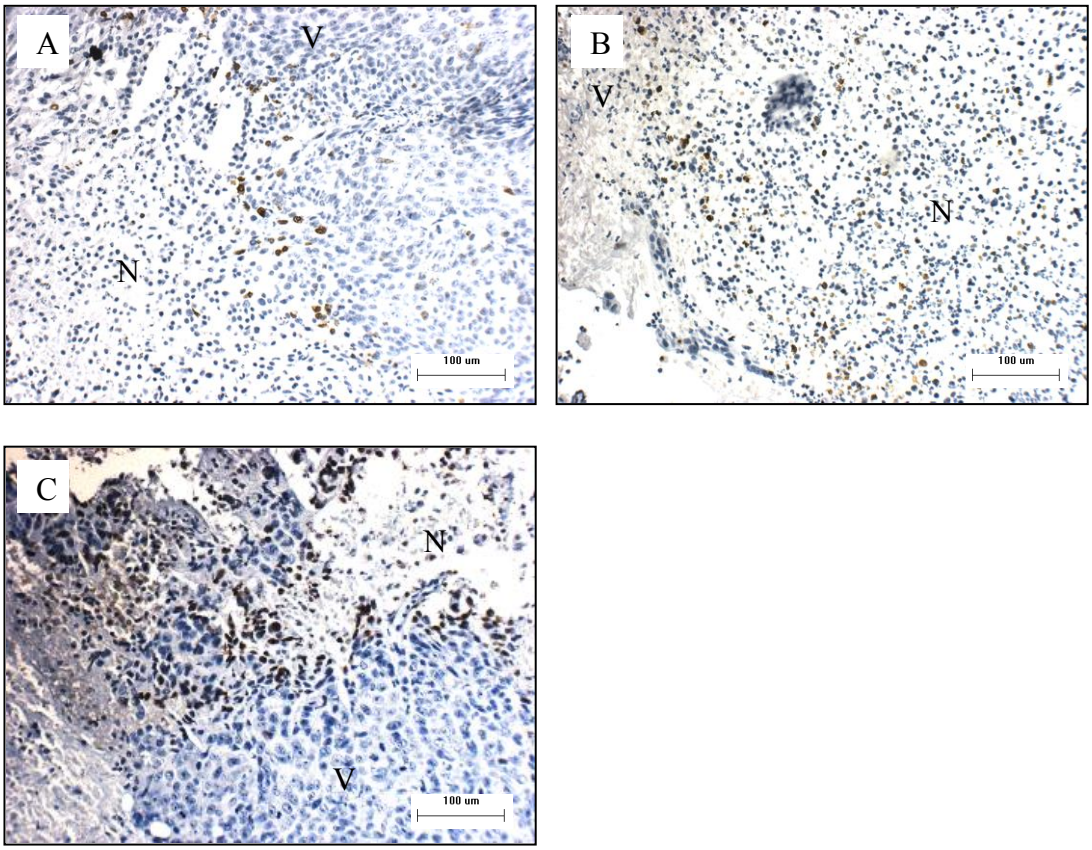


Figure 16 Expression of p-H2AX in DLD-1 Panel A, HT-29 Panel B and HCT 116 Panel C xenografts. V represents areas of viable tissue, N denotes areas of necrosis. Positive staining for p-H2AX is indicated where the nuclei of cells is brown/dark brown in appearance.

3.14 Inhibition of Autophagy in HT-29 MCTS and its effect upon cell death and senescence.

To determine what effect the inhibition of autophagy had on the cellular characteristics and the morphology of spheroids, MCTS were treated with an autophagy inhibitor

Bafilomycin A1 (10nM) for 24 hours before fixation and histology. In spheroids where autophagy has been inhibited there is a larger necrotic core and thinner viable rim, compared to size matched, untreated MCTS. As a spheroid (both treated and untreated) increases in size the ratio between the size of the necrotic core and the thickness of the viable rim increases (Figure 17). In spheroids where autophagy has been inhibited the NC/VR ratio is larger. Specifically the viable rim in untreated spheroids becomes thinner in size as the spheroids grow but it rarely goes below a thickness of 150μm for spheroids with an overall diameter of 1300μm. In contrast, when autophagy is inhibited, the viable rim of these MCTS was thinner with values as thin as 90μm recorded. A thinner viable rim could be representative of a decreased survival ability of the HT-29 cells at a shallower depth within the MCTS than previously seen. Inhibition of autophagy using Bafilomycin A1 therefore sensitises cell within the regions of the viable rim of spheroids where hypoxic cells are expected to reside leaving a thinner viable rim and an extended necrotic core.

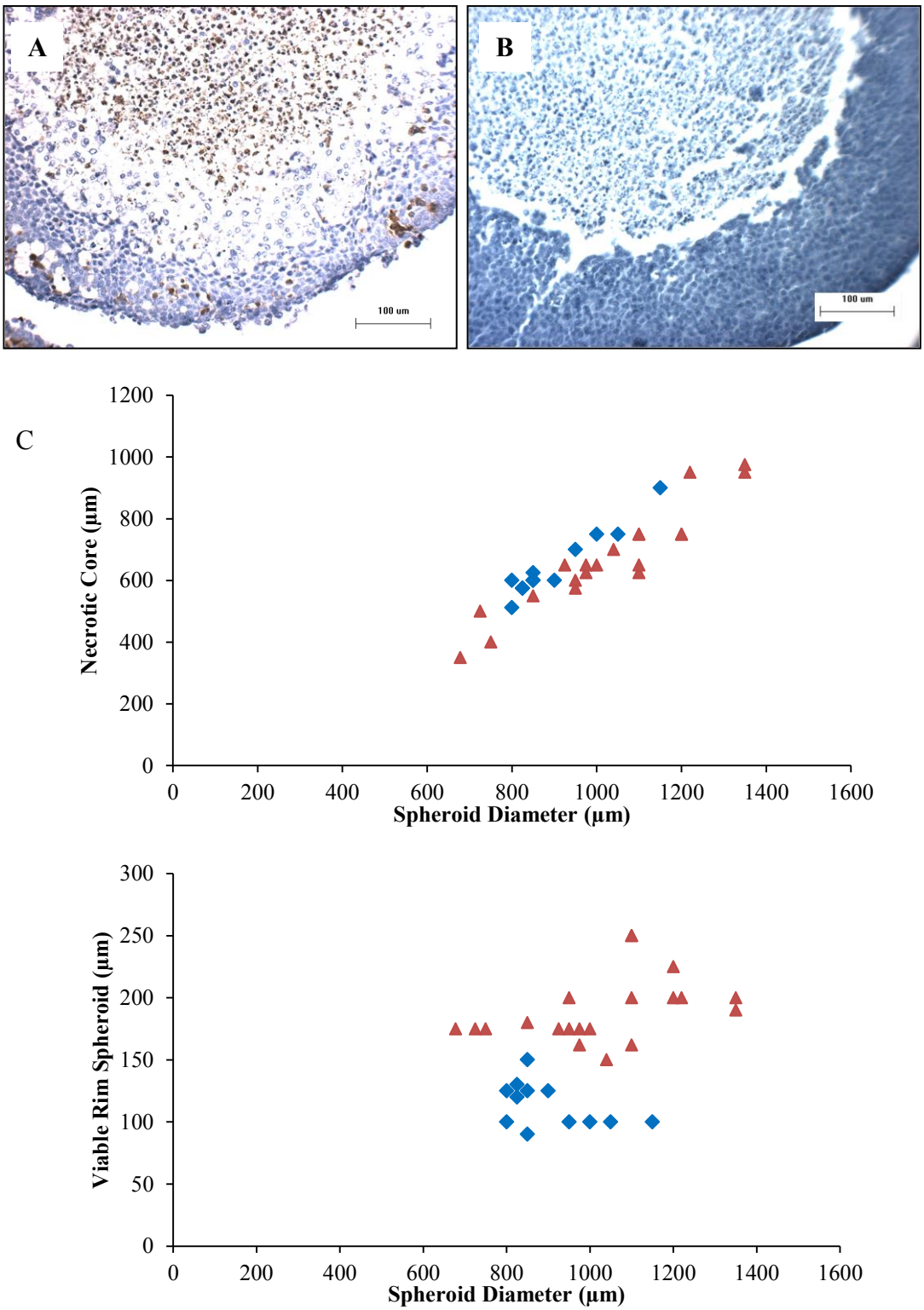


Figure 17. Data demonstrating the relationship between MCTS diameter and the relative sizes of the viable rim and necrotic core. Comparing two populations of HT-29 MCTS where one has been treated with the autophagic inhibitor Bafilomycin A1. Panel A shows cleaved caspase-3 stained HT-29 MCTS treated with BA1 with a thinning VR, Panel B shows a size matched unstained, untreated MCTS for comparison. Panel C shows the data relating to spheroid diameter and its relationship with the relative sizes of the necrotic core and viable rim.

In order to statistically analyse the two populations and determine whether they are significantly different, an ANOCOVA (analysis of covariance) was performed. This test enables the comparison of two lines of regression and determines whether the population means for the dependant variable (size of the necrotic core and viable rim respectively) differ across the levels of the independent variable (normal autophagic flux versus inhibited autophagic flux) taking into account and adjusting for the differences in the covariate (total spheroid diameter). Which in summation determines whether the adjusted group means differ significantly. The ANOCOVA performed upon this data was significant for both necrotic core and viable rim size, $F > F$ crit (Necrotic core,$18.84 > 4.2$; viable rim, $60.25>4.2$), with $p <0.001$ for the necrotic core and $p<0.0001$ for the viable rim (Table 2). The adjusted mean for the normal MCTS necrotic core size was 631μm while for the autophagy inhibited MCTS the adjusted mean was higher, at 713μm (Table 3). The adjusted means for the viable rim sizes were 188μm for normal MCTS and 117μm for the autophagy inhibited MCTS. The assumptions for the tests were met such that the covariate was linearly related to the dependant variable. Therefore the null hypothesis is rejected and the MCTS in the autophagy inhibition group have a significantly larger necrotic core and smaller viable rim than normal MCTS.

Table 2. ANOCOVA Results. A summary of the ANOCOVA results detailing the sum of squares (SS), degrees of freedom (df), mean square (MS), F value and P value. The F crit value (the inverse of the F probability) was calculated to 4.20.

Necrotic Core					
Source	**SS**	**df**	**MS**	**F**	**P**
Adjusted means	44184	1	44184	18.84	<.001
Adjusted error	68007	29	2345	-	-
Adjusted total	112191	30	-	-	-
Viable Rim					
Source	**SS**	**df**	**MS**	**F**	**P**
Adjusted means	33597	1	33597	60.25	<.0001
Adjusted error	16170	29	558	-	-
Adjusted total	49767	30	-	-	-

Table 3. Detailing the adjusted means of the dependant variable (Necrotic Core and Viable Rim diameter (μm)) for the two groups of data analysed in the ANOCOVA.

Necrotic Core			
	Normal MCTS	**Autophagy Inhibited MCTS**	**Total**
N	20	12	32
Observed Means	670	649	662
Adjusted Means	631	714	662
Viable Rim			
	Normal MCTS	**Autophagy Inhibited MCTS**	**Total**
N	20	12	32
Observed Means	190	114	161
Adjusted Means	188	117	161

3.15 Cell death and senescence in autophagy inhibited MCTS

Comparison of the expression of cell death and senescence markers in HT-29 spheroids treated with autophagy inhibitor Bafilomycin A1 showed that in the treated MCTS, cleaved caspase-3 positive cells are seen throughout the viable rim as opposed to solely in the necrotic core in untreated spheroids (Figure 18, Panel A and B). This appearance of a number of dead cells at shallower depths supports the morphological appearance of the MCTS and cells. p-H2AX expression is also altered by the inhibition of autophagy. There is, similar to the cleaved caspase-3 staining, presence of p-H2AX at shallower depths within the viable rim including cells in the outmost surface layer (Panel D). These positive cells located at the surface of the spheroid contrast with the p-H2AX positive cells in the untreated spheroids where expression is confined to the necrotic core and the first few cell layers surrounding this (Panel C). Therefore inhibition of autophagy appears to have a major impact on the fate and survival of cells grown as MCTS. These results demonstrate that autophagy is a crucial mechanism used by cells to survive poor nutrient and oxygen conditions in MCTS.

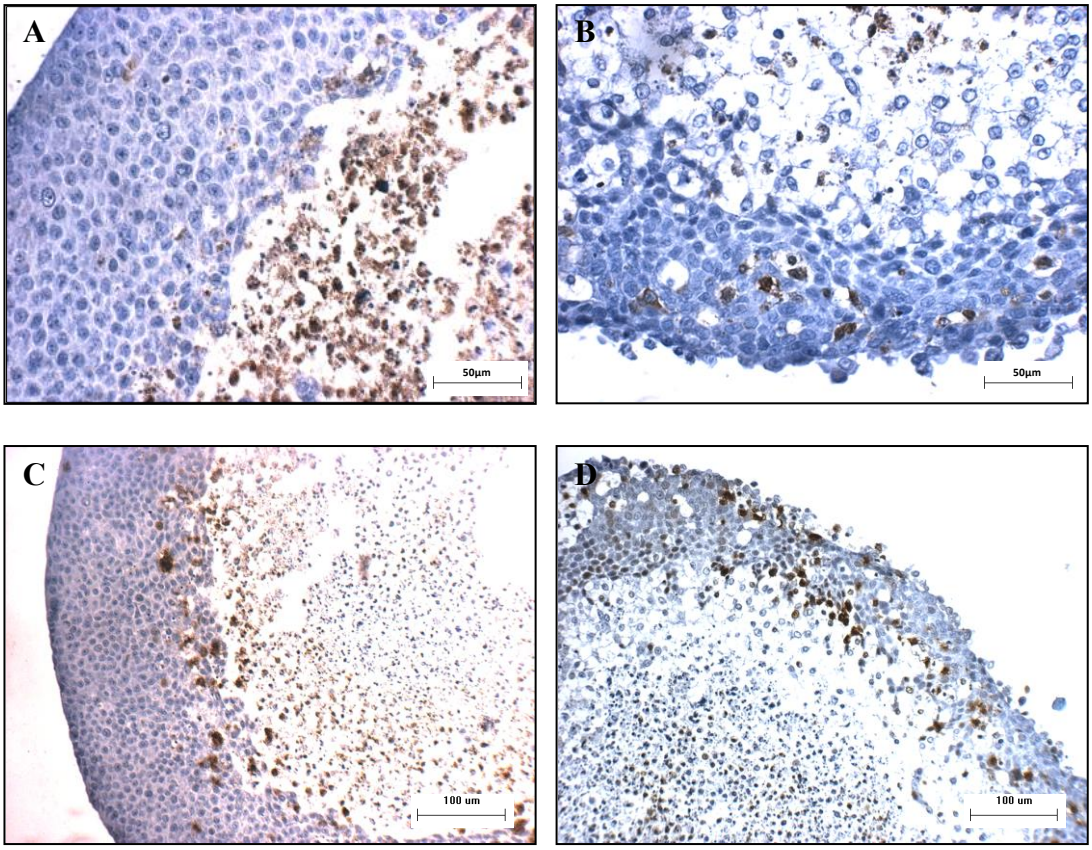


Figure 18. Immunohistochemical analysis of the differential expression of cleaved caspase-3 (CC3) and p-H2AX in untreated HT-29 MCTS and MCTS treated with autophagy inhibitor Bafilomycin A1 (10nM). Panel A shows CC3 expression in a untreated MCTS and panel B shows CC3 expression in Bafilomycin A1 treated MCTS. Panel C shows p-H2AX expression in a untreated MCTS and panel D shows expression of p-H2AX in a Bafilomycin A1 treated MCTS.

3.16 Separation of cell populations within the MCTS using Flow Cytometry

HT-29 MCTS were stained with Hoechst 33342 (1µM) for 30 minutes to generate a gradient of fluorescence. Following trypsinisation, cells were separated based on fluorescence intensity and cell size as described below. Control unstained cells (Figure 19) were analysed first to determine levels of auto fluorescence associated with the cells. When the unstained HT-29 spheroids were disaggregated into a single cell population and sorted using FACS, two distinct populations were seen (Figure 19). One consisted of larger, less granular cells from the viable rim of the spheroid that were identified with a low SSC (side scatter indicating cell granularity) and high FSC

(forward scatter indicating cell size). The other population made up the necrotic core fraction and included smaller more granular cells, with high SSC and low FSC. Both viable rim and necrotic core populations showed low levels of fluorescence.

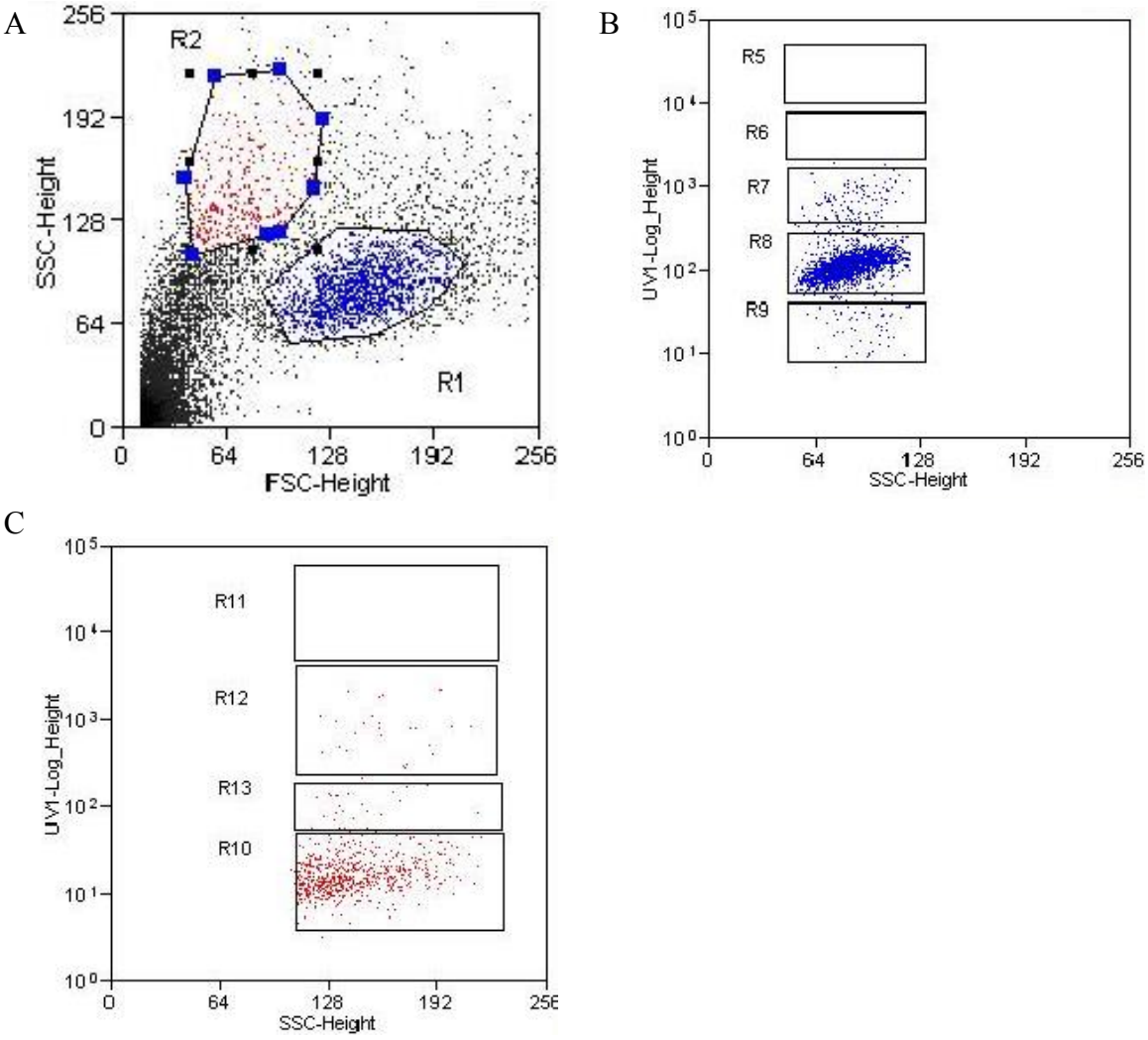


Figure 19. Control unstained MCTS FACS. Graphs representing a control unstained population of cells derived from HT-29 MCTSs showing their FSC, SSC and fluorescent signal. Panel A illustrates that MCTSs are made up of two major populations; small granular necrotic core cells characterised by their low FSC and high SSC (the population of cells in red) and large less granular viable rim cells characterised by their high FSC and low SSC (coloured blue). Panels B and C represent the low fluorescent signal observed with unstained cells.

The validity of these two groups as separate populations was supported by using the same separation parameters on a population of HT-29 MCTS which had first been stained with Hoechst 33342. Once staining was complete the MCTS were disaggregated

into a single cell population. The staining was carried out so that then cells residing in the outermost layers of the MCTS would be most strongly stained and the innermost cells would be least strongly stained. Once the single cell population was separated by SSC and FSC into the necrotic core and viable rim populations the relative fluorescent intensities from each population could be measured. The smaller more granular necrotic core population had a uniformly low fluorescent signal (see Figure 20), similar to that of unstained spheroid cells (see Figure 19). This indicated that the cells were derived from deep inside the spheroid furthest away from the source of fluorescent dye. Their fluorescent signal was expected to be low as the Hoechst would have to diffuse though many layers of cells to reach them. The larger less granular population had a range of fluorescent intensities with the highest being more than 2 orders of magnitude greater than that of unstained cells. This is consistent with this being the viable rim population with the most intensely stained cells being located in the outer most regions of the viable rim and the lesser fluorescent cells from the inner viable rim where the hypoxic cells reside.

Using the fluorescent signal of the two populations, it was possible to further spit the two groups (NC and VR) into more fractions (Figure 20). The most intensely fluorescing cells in the viable rim population were derived from the outer most layer of the spheroid, hence being the most strongly stained by the Hoechst 33342. As the fluorescent intensity decreased this corresponded to cells derived from deeper within the spheroid.

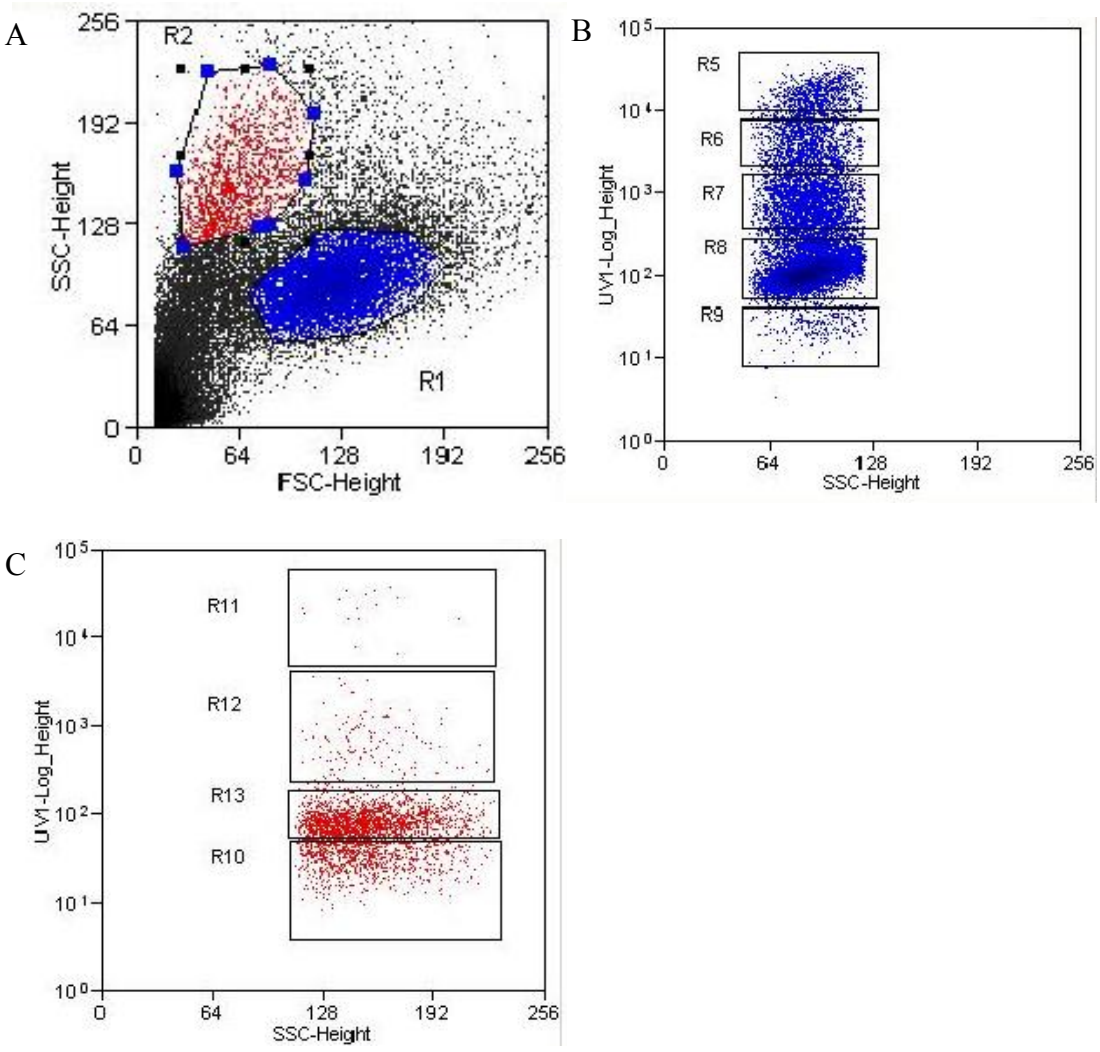


Figure 20. Hoechst 33342 stained MCTS and cell sorting. Whole intact MCTSs were stained and then disaggregated into a single cell population before analysis by flow cytometry. Cells were sorted via size, granularity and intensity of fluorescence. The blue population consists of cells from the viable rim, and as such contains the most intensely fluorescent cells. The red plot shows the cells derived from the necrotic core which in turn are the least fluorescence as it takes longer for the Hoechst to penetrate to the very centre of the spheroid.

3.17 Characterising the growth of cell populations isolated from different regions of MCTS.

The different fractions were transferred into standard cell culture conditions to determine whether they could re-establish growth when environmental conditions became favourable again and if so, could clones be generated so that the biological properties of cells derived from the necrotic core could be compared to the parental cell

line. Their plating efficiencies were measured, showing that the deeper into the spheroid from which the cells were derived, the lower their plating efficiency (
Table 4). Contrary to the perceived view that cells within the necrotic core of MCTS are dead, a significant number retain the ability to re-grow when returned to normal cell culture conditions. A plating efficiency of 5.00+/- 0.52% indicates that a substantial number of cells retain viability such that when conditions revert back to 'ideal' growth conditions, they are able to proliferate and form colonies of cells again. While the plating efficiency of cells isolated from the necrotic core is significantly lower than that of the cells taken from the viable rim the surface of the spheroids, it nevertheless confirms that viable cells exist within the necrotic core and these have the ability to re-grow when conditions becomes favourable.

Table 4. Showing the plating efficiency of the different fractions of the HT29 cells separated from a MCTS by Flow cytometry. While it is not possible from this experiment to determine the exact depths inside the MCTS from which each fraction is derived the fractions order of depths is known. R5 being derived from the surface with the most highly fluorescent population (see Figure 20) and each following fraction being derived deeper until R10 which is the innermost population of cells (R5>R6>R7>R8>R12>R13>R10).

Increasing distance from spheroid rim	Fraction Number	Plating Efficiency (% +/- SD)
Surface Layer	R5	43.33 +/- 2.07
	R6	41.67 +/- 1.67
	R7	40.00 +/- 2.19
	R8	38.33 +/- 1.33
	R12	15.00 +/- 1.05
	R13	6.67 +/- 0.52
Inner Necrotic Core	R10	5.00 +/- 0.54

3.18 Cloning of cells from HT-29 monolayers and the necrotic core of MCTS.

Cells were cloned from the necrotic core of HT-29 MCTS to enable the characterisation of the cells' growth, migration, 'stemness' and chemosensitivity.

Necrotic clones were formed from the cells collected from the necrotic core of HT29 spheroids separated via FACS. Once separated, cells were plated out in serial dilutions such that only one colony per well was obtained. Colonies formed were holoclone (round colonies composed of small compact cells that can be repeatedly passaged) in morphology, which is indicative of high replicative potential and a reduced chance of undergoing growth arrest and stem cell-like phenotype. These single colonies derived from one cell were then used to establish the necrotic core clones. Once placed into a cell culture flask these cells started growing in a similar manner to normal HT-29 cells. However, one noticeable difference was that the cells tended to grow partly as a monolayer and partly in suspension. Furthermore, these cells now had the ability to spontaneous form and maintain MCTS under normal culture conditions (i.e. with no liquid overlay). These characteristics remained for between 3 and 4 passages before the cells returned to their original way of monolayer growth.

3.19 Monolayer growth characteristics of clones derived from the necrotic core of MCTS compared to parental HT-29 cell lines.

Cell Line	Cell Doubling Time (Hours)
HT-29	25.65 +/- 1.91
NCC1	30.89 +/- 0.36*
NCC2	30.38 +/- 2.02
NCC3	26.99 +/- 0.48
NCC4	29.99 +/- 0.81*
NCC5	28.43 +/- 1.60

Table 5. Doubling times calculated for the log phase of growth for the necrotic core clones and the parental cell line, standard deviations are shown and significant statistical differences calculated using a Student's T test (<0.05) are indicated with an asterisk.

Compared to the parental HT-29 cells there are other noticeable differences in the clones, the

necrotic core clones are slower growing monolayers (Figure 21). There is a clear lag phase in the growth of all the clones and the parental cell line with the exception of NCC4 although the subsequent growth rate of this clone was still less than the parental cell line.

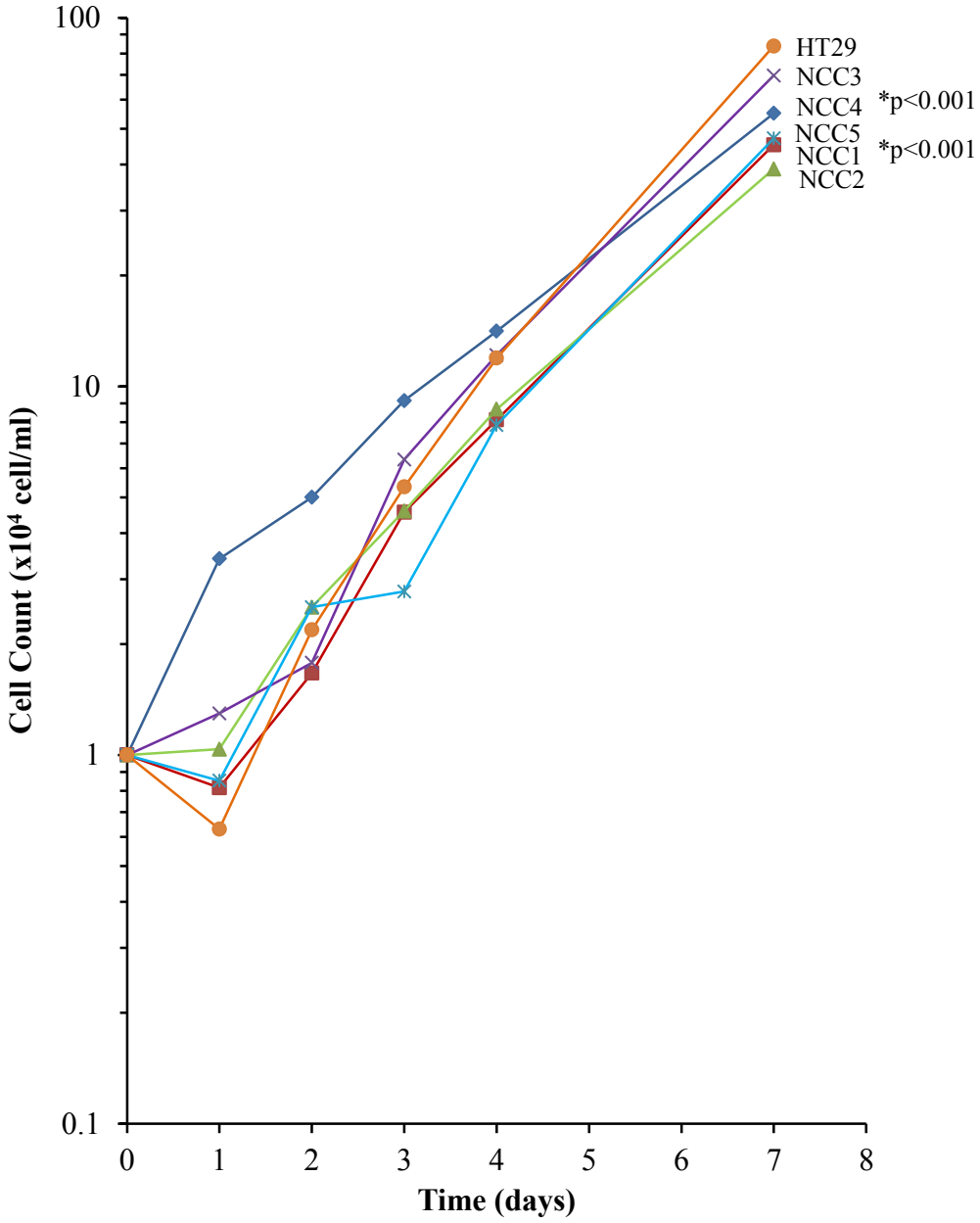


Figure 21. Necrotic Core Clone Growth Curves showing the growth of all 5 clones and the parental cell line as monolayer cultures. All growth measurements were made on cell between passage 4 and 7. Error bars are omitted in the interests of clarity. P values denote clones whose growth was statistically significant from the parental cell line as determined by ba student's t test.

When the doubling time was calculated for the log phase for each of the cell lines it was clear that all five of the clones were slower growing than the parental cell line, as all doubling rates for the clones were reduced (Figure 21). NCC1 and NCC4 were both found to have significantly lower doubling rates than the parental cell line when a Student's T test was performed.

3.20 Growth curves for spheroids derived from a necrotic core clone and the parental HT-29 cell line.

When the growth of NCC1 in spheroidal culture was compared to the parental cell line a similar trend as seen with the monolayer growth was found. NCC1 was slower growing than the parental cell line, with a statistically significant ($p<0.001$) doubling time of 80.98 hours compared to the parental 74.96 hours (see Figure 22).

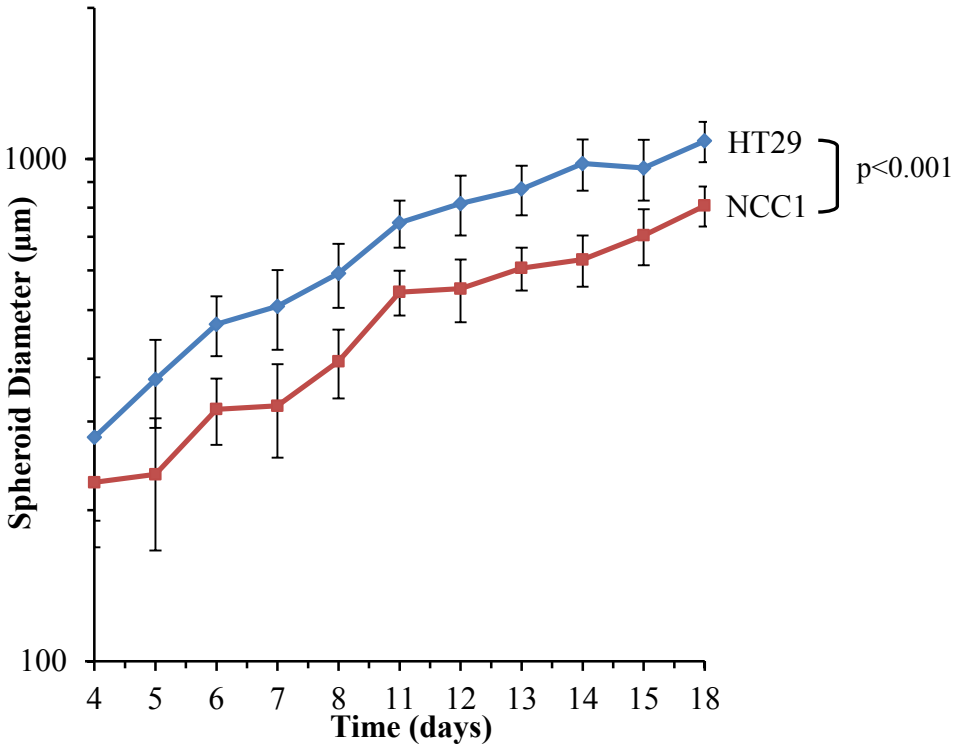


Figure 22. Growth of Necrotic Core Clone 1 as a spheroidal culture compared to the parental cell line. Measurements were taken from 20 spheroids every 2-3 days.

4 Discussion

The aims of this chapter were to establish whether there were any viable cells present in the necrotic core of MCTS and to characterise any such cells in terms of proliferative capabilities and other cellular characteristics. The key findings that have emerged from this study are that there are in fact viable cells in the necrotic core. These cells are importantly able to return to a proliferative status once the conditions become favourable. The purpose of this discussion is to interpret the key results of this chapter in the context of highlighting the novel; findings and comparing these results with previously published studies in the literature. In particular this discussion will focus on the characterisation of different cell populations within the MCTS, specifically their proliferation status and formation of necrotic areas, and the potential survival mechanisms used by the cells within the necrotic core of MCTS, including autophagy and senescence.

4.1 Culture methods for Multicellular tumour spheroid formation

Both HT-29 and DLD-1 cells lines were chosen due to their ability to form spheroids. While there are many ways to grow spheroids the preferred method to produce large numbers is to 'seed' spheroids using liquid overlay techniques and then transfer small spheroids to spinner culture vessels for continued growth.[4] The use of liquid overlay techniques to initially seed spheroids is however problematical in that spheroids of different sizes are formed due to clumping or coalescence of spheroids and it is therefore necessary to employ a spheroid sizing strategy (differential sedimentation or filtration) before transfer of spheroids to spinner flasks. In this study HT-29 cells spontaneously formed spheroids after being seeded straight into the spinner flask. As this eliminated the need to use liquid overlay followed by sizing of seeded spheroids, this represents a significant improvement to the method making it quicker and easier to

grow spheroids of a uniform size. DLD-1 cells however required the liquid overlay step before transfer to spinner flasks for the successful initiation of MCTS.

HCT116 cells were also found to form spheroids though using a different method involving seeding individual spheroids into 96 well plates base coated with 1% agarose. Spheroids were created by placing large numbers of cells in wells and allowing them to coalesce to form a spheroid. Using this method, HCT116 spheroids typically formed quicker than HT-29 and DLD-1 spheroids as they were not derived from single cells. This meant that in the HCT 116 spheroids the necrotic core forms much quicker as the spheroid does not have to grow from a single ~20 μm cell into a >500μm spheroid.

With regards to the growth characteristics of MCTS, different cell lines grow in a slightly different manner. In the case of HT-29 and DLD-1 cells, it generally takes between 24-72 hours for spheroids to first form. Necrotic cores become visible to the naked eye between 10-14 days for DLD-1 spheroids and between 7-10 days for HT-29 spheroids. The spheroids developed necrotic cores when they reached a diameter of roughly 500μm (Figure 3) and this is consistent with the diffusion distance for oxygen and nutrients widely reported in the literature.[5, 6, 7].

4.2 Characterising MCTS: Proliferation and Necrosis

The results of this study demonstrated that different cell lines can form spheroids of different maximum sizes. Typically, DLD-1 spheroids did not grow above 1000 μm before disintegrating whereas HT-29 spheroids grew to over 1500 μm in some cases (Figure 2). A common feature of all spheroid growth was the relationship between the diameter of the viable rim compared to the necrotic core. As illustrated in Figure 5, the ratio of the diameters of the viable rim to necrotic core (NC/VR) increases as the diameter of the spheroid increases. Associated with this increase in NC/VR, the thickness of the viable rim also decreases as spheroid size increases (Figure 6) and in

some regions of the spheroid, considerable thinning occurs leading to pore formation (see Figure 8). There was no evidence when the histological analysis was undertaken that this phenomenon occurred due to mechanical tearing or any form of physical trauma. Pore formation was particularly noticeable in HT-29 spheroids and when large spheroids were transferred from spinner flasks to liquid overlay cultures, extrusion of the necrotic core through the pores in the viable rim was clearly visible (Figure 8). The relationship between the thickness of the viable rim and size of necrotic core and how this varies with spheroid size has been extensively studied previously.[8, 9, 10] The results of this study are in general agreement with those reported in the literature. What has not been previously reported is the formation of pores in the viable rim and extrusion of the necrotic core. One reason why this has not been reported previously is that extrusion of the necrotic core is generally only seen in large spheroids (> 1000 μm in diameter). Spheroids of this size would be considered too large for experimental work (500 to 800 μm spheroids are typically used) and would be discarded. It is also possible that some cell lines show heterogeneity in their ability to form pores in the viable rim as HT-29 tended to form pores more readily than DLD-1 spheroids. Whatever the reason, the formation of pores and the extrusion of the necrotic core mean that any viable cells that remain within the necrotic core now have access to nutrients and oxygen leading to reactivation of cell proliferation and growth. The biological and therapeutic significance of this observation will be returned to in subsequent chapters of this thesis but for the remainder of this chapter, the discussion will focus on further characterisation of the multicellular tumour spheroid model.

When HT-29 MCTS were examined they were found to contain various sub populations of cells. By staining for the proliferation marker Ki-67 it is was clear that only the outer layers of cells within larger MCTS (>600μm) were actively proliferating (Figure 7)

which was consistent with what has been reported in the literature.[11,12,13, 14] The other cells making up the viable rim were quiescent. From looking at the morphology of the cells in the necrotic core it was evident that this area of tissue was largely dead. There was a large amount of cell debris and many cells which had become detached from their neighbouring cells.

One of the early significant findings of this study was that the necrotic core of MCTS was not made up entirely of dead cells, a finding which had only been reported once previously in the literature.[15] The survival of cells within areas of dead tissue could have significant consequences if they ever managed to return to a proliferating state. The first evidence for this was initially seen in the negative cleaved caspase-3 staining demonstrated by some of the cells found in the necrotic core of HT-29 MCTS (Figure 9). The histological staining suggested that some cells had managed to avoid cell death, or at least caspase-3 dependant cell death.

4.3 Necrosis in xenografts and human colorectal liver metastases

Colorectal xenografts from three different cells lines were examined to see whether the necrotic areas found in MCTS mirrored the morphology of necrotic tissue *in vivo*, and therefore whether the MCTS were a good model for this area of research. Areas of necrotic tissue were seen throughout the xenografts and were surrounded by areas of viable tissue similar to that of the MCTS (Figure 10) and in accordance with the literature.[16] However unlike the MCTS, expression of cleaved capase-3 was not as extensive in the necrotic areas found in xenografts. HCT 116 xenografts showed expression in only a small subset of cells and HT-29 and DLD-1 still showed less than in the HT-29 MCTS (Figure 11). Therefore MCTS do reflect xenograft tissue in terms of morphology as they both have similar areas of necrotic tissue as seen in Figure 10. However the cell death mechanism of the cells grown in MCTSs are different as seen by

the differential expression of cleaved caspase-3. When archived clinical tissue was examined, it was clear that the structure of MCTS mirrored very closely the structure of rosettes found in colorectal liver metastases (Figure 12) and as described in the literature.[17] This further validated the use of MCTS as an *in vitro* model.

4.4 Multicellular Tumour Spheroid Cell Sorting

To support the negative cleaved caspase-3 staining data (Figure 9) and to confirm that the negatively stained cells were indeed viable, cells from the different regions within MCTS, including the necrotic core were separated and collected using flow cytometry (Figure 19 and Figure 20). The Hoechst staining and FACS method used to separate the spheroids was found to be a very effective method. Whilst flow cytometry has been used previously to demonstrate that spheroids contained two distinct populations of cells, the actual process of separating the spheroids was carried out using a serial trypsinisation technique. [18] The serial trypsinisation technique involves digesting cells off the surface of the spheroids a layer at a time. Whilst this technique has definite benefits such as being simple to perform it does have some downsides. The technique relies on examining the spheroids by eye while trypsinising, and then finally using mechanical pressure to disrupt the remaining viable "shell" to release the necrotic core. There is a chance that contamination could occur between the different populations if for example a spheroid was to accidently burst during the trypsinisation process, prematurely releasing the necrotic core cells. Henceforth a more reliable technique was required. Flow cytometry is a particularly reliable technique of cell sorting as it characterises each cell individually and enables the separation of the cells based on their size and granularity. Using cell size and granularity, the population of cells within a spheroid can be separated into two groups, cells which make up the viable rim are larger and not very granular, necrotic core cells are smaller and more granular.[19] By staining

the spheroids before performing FACS it was possible to add third layer of separation to the technique. This made the results more reliable, by examining the fluorescence distribution, between the different populations separated by their size and granularity, it was possible to further validate FACS as a method of separating spheroids.[20]

Following the separation of the MCTS, cells their plating efficiency could be measured to determine how viable the cells were. The necrotic core cells were found to have a certain degree of viability. A plating efficiency of 5% while being significantly lower than the other cell populations found in the MCTS is nevertheless highly significant as it proves that some cells in the necrotic core are indeed alive. This is supported by similar research using the same cell line which found that there were various enzymes which were active within the necrotic core of MCTS.[21]

Once it was established that there were cells that were managing to survive within the necrotic core of MCTS, the next question was how do they manage to survive the poor nutrient and low oxygen environment when most other cells cannot and are they therefore different to the rest of the cell population?

4.5 DNA Damage and Senescence

Whilst it was clear from the Ki-67 staining in Figure 7 that the cells within the necrotic core were not proliferating, it was unclear whether the cells were in an active state or whether they had become senescent. Expression of the DNA damage marker which has also been found to correlate with senescence, p-H2AX, indicated that a number of cells at the peripheral edge of the necrotic core on the boundary between the necrotic core and the viable rim could be in a senescent state as well as a number of cells throughout the necrotic core (Figure 15). Senescence has previously been found to occur in MCTS though necrotic core cells have not been the focus of the studies and senescence here has not been described before.[22] Senescence therefore may be a survival mechanism

used by the cancer cells to evade cell death. Especially as cancer cells have been shown recently to have the ability to re-enter the cell cycle after a period of senescence.[23] Furthermore this evidence was mirrored *in vivo* in colorectal xenografts where similar expression was seen in necrotic areas (Figure 16). These results suggest that senescence could be used as a mechanism of cell survival in necrotic areas, therefore inhibition of the senescent phenotype might sensitise cells to cell death in these regions. Whilst the evidence for senescence is not conclusive it is clear that these cells have DNA damage which has not been previously described in the different populations within MCTS.

4.6 Autophagy

Autophagy was investigated as a mechanism by which the cells could survive the low nutrient environment within the necrotic core. Autophagy has been found to be involved in various different aspects of tumour cell biology, from drug resistance to tumourigenesis, but as an evolutionary conserved catabolic process its main role is to recycle old, damaged or unnecessary proteins in order to provide the building blocks to sustain further growth and/or survival.[24] Autophagy has been discovered to have a role in cancer stem cell maintenance in various different cancer types including pancreatic, liver and colorectal. What was found is that the cancer stem cell resistance to certain drugs such as Paclitaxel is reliant on autophagy and when this degradation pathway is inhibited the cells are sensitised to the chemotherapeutic. [25] It has also been seen that cancer stem cells, along with normal cancer cells, use autophagy to help withstand poor nutrient and low oxygen conditions. The following sections describe and discuss several key aspects related to the role autophagy plays in the necrotic core of MCTS.

4.6.1 Characterising autophagy inhibitors

A range of different autophagy inhibitors were trialled on DLD-1 cells grown on glass cover slips to determine which was the most appropriate. It was necessary to find an

autophagy inhibitor that halted the autophagic pathway in the most appropriate place, in order to measure levels of LC3-II conversion and hence autophagic flux without LC3 degradation altering protein levels. Both Chloroquine and Bafilomycin A1 increased LC3-II levels; this was expected as both Bafilomycin A1 and Chloroquine are known to inhibit the autophagic pathway downstream of autophagosome formation. In contrast 3-Methyladenine is a PI3K inhibitor that inhibits the formation of the autophagosome, leading to a reduction in the amount of punctate LC3 staining seen (Figure 13).[26] Bafilomycin A1, a V-ATPase inhibitor, prevents maturation of autophagic vacuoles.[27] It does this by blocking the fusion of the autophagosome and lysosome hence preventing the degradation of LC3. Treatment of cells undergoing autophagy with Bafilomycin A1 lead to its accumulation of LC3 in the cytoplasm, the difference in punctate LC3 staining between cells treated with and without the inhibitor indicates the extent of autophagic flux. Therefore Bafilomycin A1 was deemed suitable for determining levels of autophagic flux within cells, and this is consistent with other methods of autophagy detection described in the literatutre.[28] Chloroquine, a lysosomotropic compound which preferentially accumulates in the lysosomes preventing the fusion of the autophagosome and the lysosome similar to Bafilomycin A1, also caused an increase in LC3 levels in the cytoplasm (Figure 13). Both Bafilomycin and Chloroquine are suitable inhibitors to use for measuring autophagy.

4.6.2 Autophagy in the Necrotic Core

When the spheroid necrotic core was stained for LC3-II it was found that there was an active biological process occurring in what was originally thought of as a completely dead environment. This was seen clearly by the increase in LC3-II puncta in the necrotic core of the MCTS which were treated with an autophagy inhibitor (Figure 14). These results were confirmed by staining for a second autophagy marker Beclin-1

whose expression was also seen to be up regulated in the necrotic core. Further confirming that there were living cells within, and that autophagy was at least one possible method the cells used to survive. Active enzyme activity has previously been reported in the necrotic core of MCTS, including enzymes involved in autophagy.[29] The staining data described in Figure 14 confirms the results reported in the literature. As autophagy does not occur in normal cells under normal physiological conditions this could lead to a relatively selective way to target the cancer cells. Though as yet it is uncertain whether autophagy should be inhibited or induced, owing to its contradicting roles in cancer.[30]

4.6.3 Effect of autophagy inhibition on cell viability and DNA damage in MCTS

The effect of autophagy inhibition on HT-29 MCTS was measured to determine whether, as the LC3 staining data suggested, the cells were indeed reliant upon the process for survival within the spheroid. What was seen in the MCTS following autophagy inhibition did lend further weight to this hypothesis. In HT-29 MCTS where autophagy was inhibited the necrotic core was larger and took up a larger proportion of the whole spheroid area compared to a normal autophagy capable cell of a similar size (Figure 17). This suggests that without the help of autophagy as a survival mechanism cells within the MCTS are more sensitive to nutrient derivation. Cells closer to the surface were now staining positive for cleaved caspase-3 as the inhibition of autophagy reduced the depth threshold for cell death, in fact expression was noted throughout the viable rim, which had never been seen before in these particular MCTS (Figure 18). These results closely follow other reports of autophagy inhibition in spheroids published in the literature. However whilst autophagy inhibition has previously been shown to induce cell death in MCTS, the localisation of the cells affected in the MCTS and the effects upon viable rim and necrotic core size have not been investigated. Instead the

MCTS have been considered as one homologous population of 3D cells rather than separate populations.[31] The results highlight that in this specific situation autophagy has cancer promoting effects. Furthermore its inhibition can be used to induce cell death under conditions of nutrient deficiency specifically in the inner nutrient deprived hypoxic population of cells found surrounding the necrotic core in MCTS. Hypoxia is an established biological target in cancer therapy. Hypoxic cancer cells are known to be more resistant to radio and chemotherapy.[32] As such anti-cancer drugs have been developed to specifically target hypoxic cells by taking advantage of the specific molecular characteristics displayed by these cells. These include bioreductive prodrugs which are reduced to their active form through enzymatic reduction, in normoxic cells the active drug is then re-oxidised and deactivated, ensuring steady state levels of active drug remain low. In hypoxic cells however the active drug is not re oxidised due to the low oxygen availability allowing levels of active drug to reach critically lethal levels. Whilst these drugs are relatively selective to hypoxic cells they are subject to certain problems relating to drug penetration and speed of bioactivation.[33] Reports in the literature using MCTS and multicell layers as an *in vitro* model found that in some cases the speed of the bioreduction of the prodrug to its active form exceeded the rate of drug delivery leading to reduced efficacy in larger areas of hypoxia.[34, 35] Therefore there is a need for improved targeting of the hypoxic tumour microenvironment using different mechanisms such as that seen with autophagic inhibition. Despite 40 years of research since Sartarelli devised the term 'bioreductive activation', no hypoxia selective bioreductive drug has been approved for use in humans. Other approaches to target hypoxia aim to exploit cellular responses to hypoxia (e.eg. HIF1 and HIF1 activated pathways) but no clinically approved therapy has been developed so far. The observation here that Bafilomycin A1 kills the hypoxic fraction of cells in MCTS opens

up the possibilty of using autophagy inhibitors as hypoxia selective agents, something which has been reported previously in the literature.

As a result of autophagy inhibition DNA damage was also seen to be induced at shallower depths inside the MCTS, as shown by the p-H2AX staining (Figure 18). As cells closer to the surface are unable to withstand the nutrient and oxygen deprivation when autophagy is inhibited, this leads not only to DNA damage and possibly senescence but also eventually apoptosis. Targeting autophagy could be a viable option for eliminating cancer stem cells that either are located in nutrient poor environments, or that use autophagy to mediate a drug resistance mechanism. Inhibition of autophagy has also been shown to inhibit stem cells' self-renewal capabilities; therefore by using an autophagy inhibitor alongside a cytotoxic drug, the differentiated cells will be eliminated leaving stem cells which are no longer capable of re-growing or metastasising.[36] Inhibiting autophagy is also likely to have fewer side effects than other classic cytotoxic drugs. Drugs which inhibit autophagy include Chloroquine which is already prescribed to treat malaria.[37] Also from the results it was clear that the autophagy inhibitor used (Bafilomycin A1) was clearly capable of penetrating multiple cells layers as its effects were seen to be present in cells in the inner viable rim up to 150μm deep. One of the most important benefits of autophagy as a target for cancer therapy is that it would enable relatively specific targeting of cells which are traditionally difficult to treat due to their non-proliferative hypoxic status.[38]

4.7 Necrotic Core Clones

In order to understand how the cells survived within the necrotic core and to try and determine their biological and therapeutic significance, cells isolated from the core were cloned. The establishment of clones from within the necrotic core is a significant step towards elucidating the mechanism by which cells survive the poor conditions. The fact

that cells from within the core were viable enough to be cloned was an unexpected but significant result (Table 4). This has not been previously reported in the literature, instead the necrotic core has received little interest due to the assumption that it consists entirely of dead cells.[39]

One of the questions to be answered was concerned with how the cells managed to survive, and how they were different to the rest of the population. It was important to understand whether they were permanently or transiently different, if this make them more resistant to chemotherapeutics, and once out of the necrotic core whether they begin to behave in a similar manner to the parental cell line?

The reasoning behind this line of enquiry is to find out whether these cells within the necrotic core pose a problem when treating cancer. If an anti-proliferative drug kills the viable rim of the tumour leaving only the 'dead' cells in the necrotic core, could these cells, hidden amongst the dead, help re-grow the tumour and contribute to resistance? Also would they become permanently changed by their environment? They obviously have to adapt to survive in low oxygen and nutrient conditions but whether this leads to a permanent change is unknown. If they are changed by their time in the necrotic core, does this lead to a more aggressive phenotype, which in a clinical setting would mean a more aggressive secondary cancer? Or conversely would the only cells capable of surviving in the necrotic core be the more hardy and aggressive cancer cells, and therefore is the necrotic core an environment that selects for more aggressive cells. Many of these questions will be addressed in subsequent chapters but from the results presented here, there are some noticeable changes occurring in the necrotic core clones once they were returned to growing as monolayers. When the clones were first formed, their colony formation was holoclone in structure. Holoclone colonies are indicative of stem-like cells and their formation has been taken as a surrogate stem cell assay.[40, 41]

Once the clones were out of the necrotic core and had formed colonies, the cells continued to grow at a slower rate to normal HT-29 cells (Figure 21). In addition they were also slower when grown as MCTS (Figure 22). A slower rate of proliferation is advantageous for the survival of cancer cells when treated with many standard chemotherapeutic drugs which target actively proliferating drugs. The way in which the cells grew in culture was initially different to the parental cell line. At first the clones grew in part as a monolayer and in part as a suspension culture and only after several passages did they returned to a normal adherent cell line.

However it is important to find out whether the permanent changes observed in the necrotic core clones affect their response to chemotherapeutics. The fact that there are permanent changes in the Necrotic Core Clones compared to the parent HT-29 cells does suggest that these changes seen in cells residing in necrotic areas could have therapeutic and prognostic consequences. A slower growth/metabolic rate could possibly be one of the reasons for the necrotic core cells ability to survive within a hypoxic environment with limited nutrient availability, by slowing down cellular metabolism the cells are able to reduce their energy demands and this seems to be a characteristic that remains with the cells after conditions become favourable again. Slower rate of growth is also a characteristic of stem cells.

In conclusion, this collection of results has established that there are viable cells located within the necrotic core of HT-29 MCTS. These cells have the ability to re-grow when conditions become favourable. The next question is what are the therapeutic and biological consequences of these cells? In addition the results have demonstrated that inhibition of autophagy may be a selective way to target the nutrient deficient hypoxic cells which inhibit the hypoxic tumour microenvironment in and around areas of tumour necrosis. This avenue of research has not been pursued further in this thesis but it will

be developed further by others within the Institute of Cancer Therapeutics at the University of Bradford.

5 References

[1] McMahon KM, Volpato M, Chi HY, Musiwaro P, Poterlowicz K, Peng Y, Scally AJ, Patterson LH, Phillips RM, Sutton CW. Characterization of changes in the proteome in different regions of 3D multicell tumor spheroids. *J Proteome Res*. 2012;11(5):2863-75.

[2] McMahon KM, Volpato M, Chi HY, Musiwaro P, Poterlowicz K, Peng Y, Scally AJ, Patterson LH, Phillips RM, Sutton CW. Characterization of changes in the proteome in different regions of 3D multicell tumor spheroids. *J Proteome Res*. 2012;11(5):2863-75.

[3] Virtual Pathology at the University of Leeds. 2014. Spectrum Webscope, Slide 164463. Available at: http://slides.virtualpathology.leeds.ac.uk/ Research_4/Teaching/ Education/Postgraduate/Wednesday_Teaching/29-Feb-12/ 164463.svs/ view.apml?returnurl= http://www.virtualpathology.leeds.ac.uk/slidelibrary/ index.php. [Accessed 10.09.14].

[4] Sutherland R. M., Carlsson J., Durand R. E., Yuhas J. Spheroids in cancer research. *Cancer Res*. 1981;41:2980–2994.

[5] Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res*. 1989;49(23):6449-65.

[6] Grimes DR, Kelly C, Bloch K, Partridge M. A method for estimating the oxygen consumption rate in multicellular tumour spheroids. *J R Soc Interface*. 2014;11(92):20131124.

[7] Muschler GF, Nakamoto C, Griffith LG. Engineering principles of clinical cell-based tissue engineering. *J Bone Joint Surg Am*. 2004;86-A(7):1541-58.

[8] Freyer JP, Sutherland RM. Regulation of growth saturation and development of necrosis in EMT6/Ro multicellular spheroids by the glucose and oxygen supply. *Cancer Res*. 1986;46(7):3504-12.

[9] Tannock IF, Kopelyan I. Influence of glucose concentration on growth and formation of necrosis in spheroids derived from a human bladder cancer cell line. *Cancer Res*. 1986;46(6):3105-10.

[10] Freyer JP. Role of necrosis in regulating the growth saturation of multicellular spheroids. *Cancer Res*. 1988;48(9):2432-9.

[11] Vinci M, Gowan S, Boxall F, Patterson L, Zimmermann M, Court W, Lomas C, Mendiola M, Hardisson D, Eccles SA. Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biol.* 2012;10:29.

[12] Takagi A, Watanabe M, Ishii Y, Morita J, Hirokawa Y, Matsuzaki T, Shiraishi T. Three-dimensional cellular spheroid formation provides human prostate tumor cells with tissue-like features. *Anticancer Res*. 2007;27(1A):45-53.

[13] Laurent J, Frongia C, Cazales M, Mondesert O, Ducommun B, Lobjois V. Multicellular tumor spheroid models to explore cell cycle checkpoints in 3D. *BMC Cancer*. 2013;13:73.

[14] Mellor HR, Ferguson DJ, Callaghan R. A model of quiescent tumour microregions for evaluating multicellular resistance to chemotherapeutic drugs. *Br J Cancer*. 2005;93(3):302-9.

[15] McMahon KM, Volpato M, Chi HY, Musiwaro P, Poterlowicz K, Peng Y, Scally AJ, Patterson LH, Phillips RM, Sutton CW. Characterization of changes in the proteome in different regions of 3D multicell tumor spheroids. *J Proteome Res*. 2012;11(5):2863-75.

[16] Leith JT, Michelson S. Changes in the extents of viable and necrotic tissue, interstitial fluid pressure, and proliferation kinetics in clone. A human colon tumour xenografts as a function of tumour size. *Cell Prolif.* 1994;27:723–739.

[17] Sundaresan M, Lyons B, Akosa AB. 'Solitary' necrotic nodules of the liver: an aetiology reaffirmed. *Gut*. 1991;32(11):1378-80.

[18] McMahon KM, Volpato M, Chi HY, Musiwaro P, Poterlowicz K, Peng Y, Scally AJ, Patterson LH, Phillips RM, Sutton CW. Characterization of changes in the proteome in different regions of 3D multicell tumor spheroids. *J Proteome Res*. 2012;11(5):2863-75.

[19] McMahon KM, Volpato M, Chi HY, Musiwaro P, Poterlowicz K, Peng Y, Scally AJ, Patterson LH, Phillips RM, Sutton CW. Characterization of changes in the proteome in different regions of 3D multicell tumor spheroids. *J Proteome Res*. 2012;11(5):2863-75.

[20] Durand RE, Chaplin DJ, Olive PL. Cell sorting with Hoechst or carbocyanine dyes as perfusion probes in spheroids and tumors. *Methods Cell Biol*. 1990;33:509-18.

[21] McMahon KM, Volpato M, Chi HY, Musiwaro P, Poterlowicz K, Peng Y, Scally AJ, Patterson LH, Phillips RM, Sutton CW. Characterization of changes in the proteome in different regions of 3D multicell tumor spheroids. *J Proteome Res*. 2012;11(5):2863-75.

[22] Zhou Y, Arai T, Horiguchi Y, Ino K, Matsue T, Shiku H. Multiparameter analyses of three-dimensionally cultured tumor spheroids based on respiratory activity and comprehensive gene expression profiles. *Anal Biochem*. 2013;439(2):187-93.

[23] Roberson RS, Kussick SJ, Vallieres E, Chen SY, Wu DY. Escape from therapy-induced accelerated cellular senescence in p53-null lung cancer cells and in human lung cancers. *Cancer Res*. 2005;65(7):2795-803.

[24] Mathew R, White E. Autophagy, stress, and cancer metabolism: what doesn't kill you makes you stronger. *Cold Spring Harb Symp Quant Biol.* 2011;76:389-96.

[25] Wu S, Wang X, Chen J, Chen Y. Autophagy of cancer stem cells is involved with chemoresistance of colon cancer cells. *Biochem Biophys Res Commun*. 2013;434(4):898-903.

[26] Mizushima N, Yoshimorim T, Levine B. Methods in Mammalian Autophagy Research. *Cell*. 2010;140(3):313–326.

[27] Barth S, Glick D, Macleod KF. Autophagy: assays and artifacts. *J Pathol*. 2010;221(2):117-24.

[28] Klionsky DJ et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy*. 2008;4(2):151-75.

[29] McMahon KM, Volpato M, Chi HY, Musiwaro P, Poterlowicz K, Peng Y, Scally AJ, Patterson LH, Phillips RM, Sutton CW. Characterization of changes in the proteome in different regions of 3D multicell tumor spheroids. *J Proteome Res*. 2012;11(5):2863-75.

[30] Zhou S, Zhao L, Kuang M, Zhang B, Liang Z, Yi T, Wei Y, Zhao X. Autophagy in tumorigenesis and cancer therapy: Dr. Jekyll or Mr. Hyde? *Cancer Lett.* 2012;323(2):115-27.

[31] Ma XH, Piao S, Wang D, McAfee QW, Nathanson KL, Lum JJ, Li LZ, Amaravadi RK. Measurements of tumor cell autophagy predict invasiveness, resistance to chemotherapy, and survival in melanoma. *Clin Cancer Res.* 2011;17(10):3478-89.

[32] Brown JM. The hypoxic cell: a target for selective cancer therapy--eighteenth Bruce F. Cain Memorial Award lecture. *Cancer Res*. 1999;59(23):5863-70.

[33] Wilson WR, Hay MP. Targeting hypoxia in cancer therapy. *Nat Rev Cancer.* 2011;11(6):393-410.

[34] Durand RE, Olive PL. Evaluation of bioreductive drugs in multicell spheroids. *Int J Radiat Oncol Biol Phys.* 1992;22(4):689-92.

[35] Kyle AH, Minchinton A. Measurement of delivery and metabolism of tirapazamine to tumour tissue using the multilayered cell culture model. *Cancer Chemother Pharmacol*. 1999;43(3):213-20.

[36] Rausch V, Liu L, Apel A, Rettig T, Gladkich J, Labsch S, Kallifatidis G, Kaczorowski A, Groth A, Gross W, Gebhard MM, Schemmer P, Werner J, Salnikov AV, Zentgraf H, Büchler MW, Herr I. Autophagy mediates survival of pancreatic tumour-initiating cells in a hypoxic microenvironment. *J Pathol*. 2012;227(3):325-35.

[37] White NJ. The role of anti-malarial drugs in eliminating malaria. *Malar J*. 2008;7 Suppl 1:S8.

[38] Cosse JP, Michiels C.Tumour hypoxia affects the responsiveness of cancer cells to chemotherapy and promotes cancer progression. *Anticancer Agents Med Chem.* 2008;8(7):790-7.

[39] Milotti E, Chignola R. Emergent properties of tumor microenvironment in a real-life model of multicell tumor spheroids. *PLoS One*. 2010;5(11):e13942.

[40] Barrandon Y, Green H. Three clonal types of keratinocyte with different capacities for multiplication. *Proc Natl Acad Sci U S A.* 1987;84(8):2302-6.

[41] Bortolomai I, Canevari S, Facetti I, De Cecco L, Castellano G, Zacchetti A, Alison MR, Miotti S. Tumor initiating cells: development and critical characterization of a model derived from the A431 carcinoma cell line forming spheres in suspension. *Cell Cycle*. 2010;9(6):1194-206.

Chapter 2: Biological Implications of viable cells within the necrotic core of MCTS

1 Introduction

Following the identification that there are viable cells in the necrotic core of MCTS, different survival mechanisms used by the cells were investigated to understand why some cells managed to survive. EMT confers a certain survival advantage to cells as it makes them resistant to anoikis and therefore could be responsible for the survival of cells within the liquid like centre of the necrotic core. Expression of markers associated with EMT have, like necrosis, been found to be an indicator of poor prognosis in a variety of different cancer types.[1, 2, 3] Therefore by establishing whether the viable cells in the necrotic core have undergone EMT this may help further understand the link between necrosis and poor prognosis. Stem cells are also known to survive anchorage independent conditions and have increased survival capabilities in response to stress.[4, 5] Furthermore stem cells have been implicated in tumour progression, recurrence and metastasis.[6] Senescence is another cellular response to stress, while originally believed to be irreversible cancer cells have been shown to be capable of reversing the process and returning to a proliferative state.[7, 8] Senescent cells by the nature of their non-proliferative status are resistant to many conventional chemotherapeutics, however as senescence was not believed to be reversible the senescent cells themselves seemingly had no relevance when trying to understand tumour progression. In cancer the cells could be using senescence as a survival strategy therefore making it a possible target. Tumours are known to contain heterogeneous populations of cells which include cells with a variety of different characteristics. It is important to understand whether the necrotic core selects for cells which are more aggressive and resistant and if so to find ways to eliminate the cells. The specific aims of this chapter are to characterise the necrotic core of HT-29 MCTS and the necrotic core clones in terms of their migration, proliferation, senescence, EMT status, stem cell characteristics and *in vivo* growth.

2 Materials and Methods

Following the growth of MCTS as described in Chapter 1 the different populations could then be examined in situ using different cell staining and immunohistochemical techniques. Once physically separated the different populations can be analysed by western blotting, and the necrotic core clones can be examined using different functional assays.

2.1 Haematoxylin and Eosin Staining of MCTS

Sections were de-paraffinised with xylene (3x5 minutes) and rehydrated using ethanol (3x2 minutes) before being washed in running tap water for 5 minutes. Sections were then stained with Mayer's haematoxylin (Sigma Aldrich, St. Louis, MO, USA) for 10 minutes. Excess stain was removed from the section by soaking in acid alcohol for 60 seconds before rinsing in running tap water for 5mins and then immersing in Scott's Tap Water for 60 seconds to allow the colour to develop. Sections were counterstained in Eosin (Sigma Aldrich, St. Louis, MO, USA) for 60 seconds before a final wash in running tap water. Finally the sections were dehydrated using sequential ethanol (1x2minutes, 2x1minute) and xylene (3x1minute) series and mounted using diphenylxylene (BDH, Pool, UK).

2.2 Immunohistochemistry

Sections were de-paraffinised with xylene (3x5 minutes) and rehydrated using ethanol (3x2 minutes) before being washed in running tap water for 5 minutes. If necessary, antigen retrieval was then carried out by heating slides in antigen retrieval solution (Table 1) in a microwave for a specific length of time before cooling. Slides were rinsed

in tris-buffered saline (TBS) before endogenous peroxidise activity was blocked for 10 minutes at room temperature. Following this, further blocking steps were carried out depending on the primary antibody used (Table 1). Non-specific binding was blocked by incubating the sections with Antibody Diluent containing Bovine Serum Albumin (Invitrogen, Camarillo, CA, USA) immediately prior to the application of the primary antibody. The sections were incubated with 100µl of the primary antibody diluted in Antibody Diluent in a humidified chamber. After incubation slides were washed in TBS containing 10% (v/v) Tween-20 (TBST) (2x5 minutes) and TBS (1x5 minutes) before the application of 2 drops of labelled polymer-HRP secondary antibody, and then incubated in a humidified chamber at room temperature for 1 hour. Following this, slides were again washed in TBST (2x5 minutes) and TBS (1x5 minutes). Sections were then incubated with 3,3-diaminobenzidine (DAB, DAKO North America Inc, CA, USA) for 10 minutes at room temperature, where the horseradish peroxidise breaks down the DAB via an oxidation reaction to produce a brown precipitate at the location of the antigen. Sections were washed in running tap water for 5 minutes before being counterstained using Mayer's haematoxylin (Sigma Aldrich, St. Louis, MO, USA) for 30 seconds, rinsed in tap water for 60 seconds, Scott's tap water for a further 60 seconds and finally tap water again for 60 seconds. Sections were then dehydrated using sequential ethanol (1x2mins, 2x1min) and xylene (3x1min) series and finally mounted using diphenylxylene (BDH, Dorset). A list of all antibodies used and specific conditions of IHC are presented in table 1.

Table 1. Listing all antibodies used for immunohistochemistry and the specific conditions used for antibody incubation, antigen retrieval and blocking.

Primary Antibody	Dilution	Incubation	Antigen Retrieval	Blocking Reagents	Secondary Antibody
Anti-Cleaved Caspase-3 antibody (Asp175, rabbit monoclonal anti-human Cleaved Caspase-3 IgG; New England BioLabs, MA, USA)	1:800	4°C Overnight	10 min Citrate Buffer (10mM, pH 6.0) 20 min cooling	5min Envision H_2O_2, 2 hours 4% (v/v in TBS) Normal Goat Serum	Labelled polymer-HRP anti-rabbit (K4010, EnVision Kit, DAKO)
Anti-H2AX antibody (20E3, rabbit monoclonal anti Phospho-Histone H2A.X IgG; Cell Signalling, MA, USA)	1:400	1 hour RT	10 min Citrate Buffer (10mM, pH 6.0) 20 min cooling	3% H_2O_2 block, 10min Zymed Antibody Diluent	Labelled polymer-HRP anti-rabbit (K4006, EnVision Kit, DAKO)
Anti-Snail (2G11, mouse monoclonal anti human snail homolog 1 IgG; Novus Biologicals, CO, USA; 0.1mg)	1:100	1 hour RT	None	3% H_2O_2 block, 10min Zymed Antibody Diluent	Labelled polymer-HRP anti-mouse (K4010, EnVision Kit, DAKO)
Anti-Twist1 antibody(rabbit polyclonal anti human Twist related protein 1; Merck Millipore, MA, USA; 1 mg/ml)	1:500	1 hour RT	None	3% H_2O_2 block, 10min Zymed Antibody Diluent	Labelled polymer-HRP anti-rabbit (K4010, EnVision Kit, DAKO)

Table 1 Continued

Primary Antibody	Dilution	Incubation	Antigen Retrieval	Blocking Reagents	Secondary Antibody
Anti- CD24 antibody (rabbit polyclonal anti human CD24 IgG; Abcam, Cambridge, UK; 1mg/ml)	1:200	1 hour RT	10 min Citrate Buffer (10mM, pH 6.0) 20 min cooling	0.3% H_2O_2 block, 10min Zymed Antibody Diluent	Labelled polymer-HRP anti-rabbit (K4010, EnVision Kit, DAKO)
Anti-CD44 antibody (EPR1013Y, rabbit monoclonal anti human CD44 IgG; Abcam, Cambridge, UK; 1mg/ml)	1:100	1 hour RT	20 min EDTA Buffer (1M, pH 8.0) 20 min cooling	0.3% H_2O_2 block, 10min Zymed Antibody Diluent	Labelled polymer-HRP anti-rat (K4010, EnVision Kit, DAKO)
Anti-CD133 antibody (rabbit polyclonal anti human CD133 IgG; Biorbyt, CA, USA; 1mg/ml)	1:800	1 hour RT	10 min Citrate Buffer (10mM, pH 6.0) 20 min cooling	0.3% H_2O_2 block, 10min Zymed Antibody Diluent	Labelled polymer-HRP anti-rabbit (K4010, EnVision Kit, DAKO)
Anti-E-Cadherin (EP700Y, rabbit monoclonal anti human E-Cadherin IgG, Merck Millipore, MA, USA)	1:400	1 hour RT	10 min Citrate Buffer (10mM, pH 6.0) 20 min cooling	0.3% H_2O_2 block, 10min Zymed Antibody Diluent	Labelled polymer-HRP anti-rabbit (K4010, EnVision Kit, DAKO)
Anti-N-Cadherin (EPR1792Y, rabbit monoclonal anti human N-Cadherin IgG; Merck Millipore, MA, USA)	1:100	1 hour RT	20 min EDTA Buffer (1M, pH 8.0) 20 min cooling	0.3% H_2O_2 block, 10min Zymed Antibody Diluent	Labelled polymer-HRP anti-rabbit (K4010, EnVision Kit, DAKO)

2.3 Western Blotting

The NCCs and cells from HCT 116, DLD-1 and HT-29 MCTS were investigated for expression of 3 proteins listed in Table 2. NCC cells were collected by mechanical scrapping, washing with PBS then centrifuged at 1000*g* for 5 minutes to form a pellet. MCTS cells were collected using Trypsin to digest the surface layer cells, and a pestle to mechanically break open the MCTS to release the necrotic core cells. Cells were then washed in PBS and pelleted using centrifugal force as before.

Table 2. Primary and secondary antibodies and their corresponding experimental conditions used in Western Blotting.

Primary Antibody	Antibody dilution and experimental conditions	Secondary Antibody
Anti-E-Cadherin (EP700Y, rabbit monoclonal anti human E-Cadherin IgG, Merck Millipore, MA, USA)	1 : 30,000; overnight at 4^{o}C	HRP-conjugated goat anti-rabbit secondary antibody (P0448, DAKO, Agilent Technologies, CA, USA)
Anti-N-Cadherin (EPR1792Y, rabbit monoclonal anti human N-Cadherin IgG; Merck Millipore, MA, USA)	1 : 30,000; overnight at 4^{o}C	HRP-conjugated goat anti-rabbit secondary antibody (P0448, DAKO, Agilent Technologies, CA, USA)
Anti-Snail (2G11, mouse monoclonal anti human snail homolog 1 IgG; Novus Biologicals, CO, USA; 0.1mg)	1 : 500; overnight at 4^{o}C	HRP-conjugated goat anti-mouse secondary antibody (P0447, DAKO, Agilent Technologies, CA, USA)
Anti-β-Actin (rabbit polyclonal anti human beta Actin IgG, Abcam, Cambridge, UK; 1mg/ml)	1 : 15,000; one hour at 20^{o}C	HRP-conjugated goat anti-rabbit secondary antibody (P0448, DAKO, Agilent Technologies, CA, USA)

2.3.1 Cell Lysis

Cell pellets were lysed in protein extraction buffer (7M urea, 4% CHAPS, 0.1% SDS, 0.05% Sodium Deoxycholate and 1X PIC (Protease inhibitor complex, Roche,

Indianapolis, USA) with EDTA) by incubating the cell pellet on ice for 25 minutes in approximately 3x the pellet volume of lysis buffer. The pellet was then sonicated twice at 50% power for 10 seconds using a SH70A sonicator (Scientific Laboratory Supplies,Nottingham, U.K.). with a 2 minute incubation interval on ice. Following this the sample was centrifuged at 12,000*g* for 10 minutes at 4^{o}C and the supernatant collected. After this the protein concentration was determined using the Bradford assay.

2.3.2 Bradford Assay

The Bradford assay (Bradford, 1976) was used to measure the total protein concentration in each sample, relying on the linear relationship between the amount of Coomassie dye bound to the proteins present in the sample and the absorption. Before the amount of protein in a sample can be calculated a standard curve must first be created as follows .Bovine Serum Albumin was dissolved in HPLC (1mg/ml) and this stock was then serially diluted 4 times to give 6 decreasing concentrations of BSA (including 1 blank). 50µl of BSA sample was then added to the sample tube and 1.5ml of Bradford reagent was added and left to incubate at room temperature for 15min. All the BSA samples were then analysed on a spectrophotometer using SkanIt software (ThermoFisherScientific) to measure the absorbance of the solution at 595nm. The concentration of BSA was plotted against the absorbance of the solutions producing a calibration curve.

Once the calibration curve has been created the same procedure is carried out on the lysed cell samples. 5µl of sample is added to 45µl HPLC water before 1.5ml of Bradford reagent is added and incubated at room temperature for 15 minutes. The absorbance of each sample and BSA dilution was measured using a spectrophotometer at 595nm and the protein concentration of the samples was then calculated using the standard curve. Samples where absorbance values fall outside the linear range of the

calibration curve were diluted to ensure that they were within the linear range and the values for protein concentration were corrected for the dilution factor.

2.3.3 Polyacrylamide Gel Electrophoresis

SDS Loading buffer [5 X, Bromophenol blue (0.25%), DTT (dithiothreitol; 0.5 M), Glycerol (50%), SDS (sodium dodecyl sulfate; 10%), Tris-Cl (0.25 M, pH 6.8)] was added to all samples (2:1) and loaded onto each lane (equivalent of 50µg of protein per well) of an 10% polyacrylamide gel with 5% stacking gel. Then 5µl of protein ladder (PageRuler Plus Prestained Protein Ladder, ThermoScientific, Leicestershire, UK) was loaded into the final well. Electrophoresis was carried out using a Mini Trans-Blot system (Bio-Rad, Hertfordshire, UK) in a tris-glycine electrophoresis buffer (25mM tris base, 250mM glycine, 0.1% w/v SDS, pH 8.3) with a voltage of 60V for the first 20 minutes as the protein moved through the stacking gel after which, the voltage was increased to 100V for a further 45 minutes.

2.3.4 Electroblotting Transfer

After separation by gel electrophoresis proteins were transferred onto nitrocellulose membrane (Amersham Hybond ECL, GE Healthcare Lifesciences, Buckinghamshire, UK). The electrophoretic transfer was performed at 80V for 2½ hours using Transfer Buffer (39 mM glycine, 48 mM Tris-HCl, 0.037% SDS, 20% methanol) in the Mini Trans-Blot system (Bio-Rad, Hertfordshire, UK).

2.3.5 Protein Detection

Membranes were then blocked in 5% (w/v) non fate dried milk in TBS for 2 hours then incubated with primary antibodies (see Table 2) overnight at 4^{o}C. After washing in TBST (3x15min) membranes were incubated with HRP-conjugated secondary antibody

(1 in 500 dilution) for 1 hour at room temperature (Sigma Aldrich, St Louis, MO, USA) before detection with chemoluminescent HRP substrate (Western Lightening Plus ECL, Perkin Elmer, Massachusetts, USA). Membranes were placed in a plastic envelope and exposed to autoradiography film for between 1 and 10 minutes. Blots were then stripped with Restore Western Blot Stripping Buffer (ThermoScientific, Leicestershire, UK) for 20 minutes, washed in TBS (1x15 minutes) and re-probed with β-Actin primary antibody to confirm equal protein loading. All experiments were repeated 3 times and blots analysed with GelAnalyzer (GelAnalyzer 2010a, http://www.gelanalyzer.com/). Band intensities were normalized with regards to the β-Actin band densities.

2.4 Wound Healing Assay

In order to determine the individual motilities of the NCCs and parental HT-29 cell line, a wound healing assay was performed. Cells were plated in 6 well plates at concentrations of $3x10^6$ cells/well and incubated in 5ml of media for 48 hours to allow cells to adhere and form a confluent monolayer. The monolayers were then 'wounded' using a 100µl tip and washed twice with Hanks Balanced Salt Solution before incubating in fresh media. Media was changed every 24 hours. The wounds were photographed at 0, 24 and 48 hours post 'wounding'. The width of the wound was determined using Image J analysing software. The rate of migration was determined by calculating the rate at which the wound grew smaller over time:

$$migration = \frac{(width\ at\ 0hrs) - (width\ at\ x\ hrs)}{time(x)}$$

2.5 Sphere Formation Assay

To determine the stem cell functionality of the NCCs, a sphere formation assay was conducted. NCC and parental HT-29 cells were seeded at a density of 250 cell/well on agar coated 24 well plates in 300µl stem cell medium (DMEM/F12 serum free medium supplemented with 20ng/ml EGF, 1x B27, 1x N2, 1% sodium pyruvate).[9] Cells were then allowed to grow in anchorage independence in the above medium for 14 days, at which point the number of wells containing spherical organoids was counted. Sphere-forming efficiency (SFE) was calculated using the following formula:

$$SFE = \frac{number\ of\ spheres \times average\ sphere\ diameter}{number\ of\ cells\ seeded}$$

2.6 In Vivo Analysis of HT-29 and Necrotic Core Clone 4

To determine if any of the NCCs could form a hetergeneous tumour *in vivo* similar to the parental cell line, a xenografts formation experiment was undertaken. HT-29 and Necrotic Core Clone 4 monolayers in the exponential growth phase were harvested using trypsin. 3 male nude mice for each cell line were injected subcutaneously with 100µl of a $1x10^7$ cell/ml solution into each flank. The mice were then monitored for tumour growth and measurements made using callipers, tumour volume was determined using the following formula:

$$tumour\ volume = \frac{(length \times width^2)}{2}$$

3 Results

3.2 E-Cadherin and N-Cadherin staining to determine Epithelial-Mesenchymal Transition within the Necrotic Core

To understand how some cells managed to survive and migrate out of the necrotic core, expression of markers of the Epithelial-Mesenchymal Transition (EMT) were determined. As the necrotic core of the MCTS exists in a 'liquid like' state it was hypothesised that in order to avoid anoikis, the cells must have undergone the EMT. To determine this, HT-29 MCTS sections were stained for E-Cadherin and N-Cadherin protein expression. The expression of E-Cadherin was found throughout the MCTS (Figure 1, Panel A and B) although some cells within the necrotic core showed no staining (Figure 1, Panel A). N-Cadherin expression was seen only in a subset of cells within the necrotic core (Figure 1, Panel C), the rest of the MCTS did not express N-Cadherin **(**Figure 1**,** Panel D). The clusters of cells expressing N-Cadherin looked similar in terms of morphology to the cells negative for Cleaved Caspase-3 (Chapter 1, Figure 8). These cells appeared intact and had a small, condensed but not fragmented nucleus with a clearly defined cytoplasm.

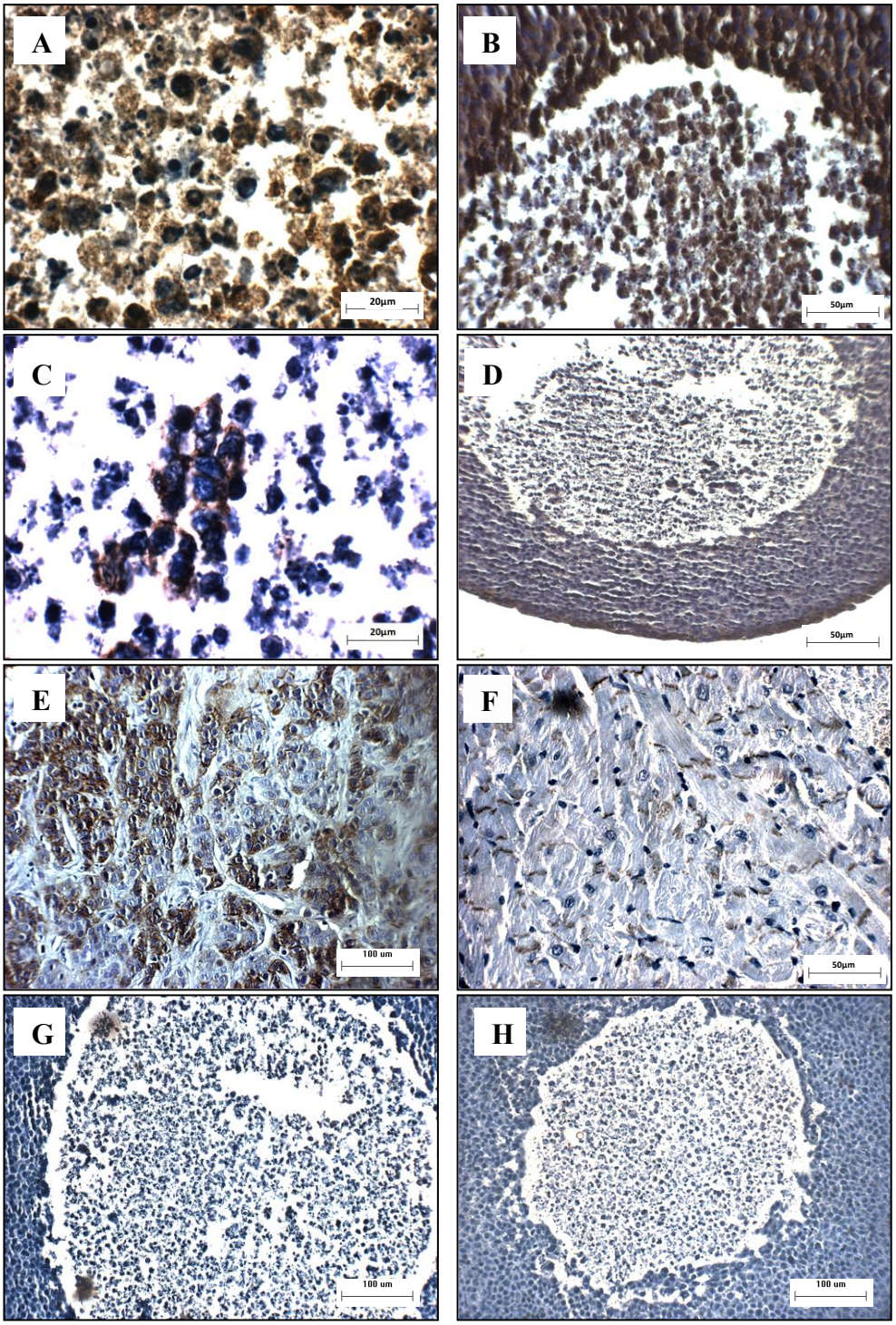


Figure 1. EMT marker staining in the necrotic core of HT-29 MCTS. Panel A shows the loss of expression of E-Cadherin in a single cell in the necrotic core. Panel B shows the positive expression of E-Cadherin throughout the MCTS. Panel C shows the expression of N-Cadherin in a cluster of cells in the necrotic core. Panel D shows the lack of N-Cadherin expression in the rest of the MCTS. Panel E shows membranous E-Cadherin staining in the positive control MCF-7 xenograft. Panel F shows the positive control used for N-Cadherin staining, mouse myocardium tissue. Panel G and H show the primary antibody control for E-Cadherin and N-Cadherin respectively.

3.2 Snail and Twist Expression within MCTS

Snail and Twist are known transcriptional repressors of E-Cadherin and markers of the EMT process and their expression was determined in HT-29 MCTS. As shown in Figure 2, Snail was expressed in the viable rim but expression was not consistent across the viable rim. The expression of Snail was observed in the cells that resided some distance (~100μm) from the surface of the MCTS in the inner viable rim (hypoxic fraction). When the necrotic core was examined, expression was seen in a small number of cells throughout the core. Both the positive cells in the necrotic core and a proportion of those in the viable rim were found to have nuclear expression of Snail, which is known to be linked to the EMT. Though a number of cells within the viable rim were found to have a cytoplasmic distribution of the protein, tumour populations have been found to contain cells with both patterns of protein localisation.[10] Nuclear rather than cytoplasmic localisation of the protein has been found to be a poor prognostic indicator.[11, 12] These necrotic cells share the same morphology as the Cleaved Caspase-3 negative and N-Cadherin positive cells in that they looked like intact cells albeit with a reduced cytoplasm and denser nuclei. This contrasts sharply with the majority of cells in the necrotic core that appeared fragmented. Twist was found to be expressed by the majority of the cells present in all areas of the MCTS.

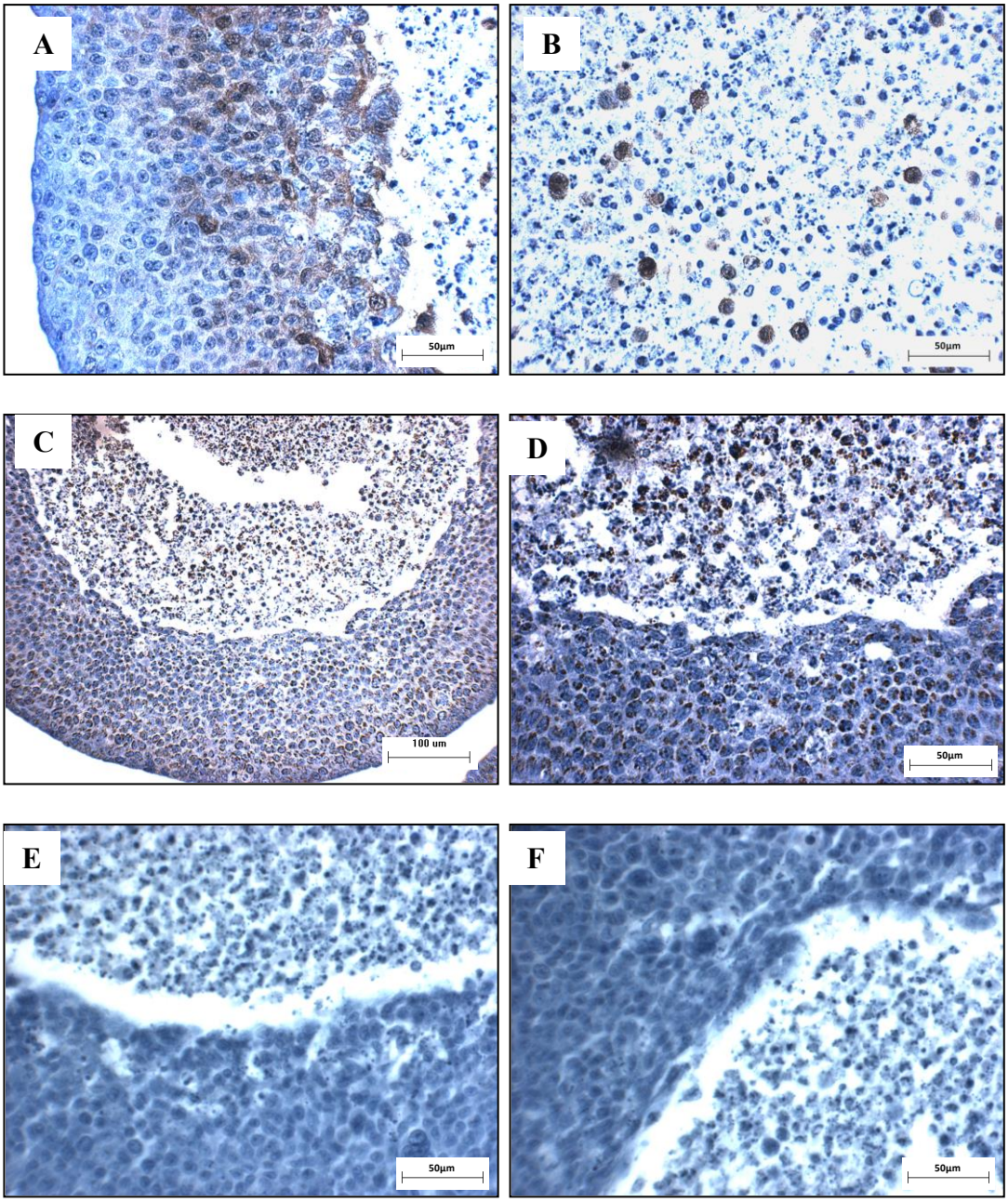


Figure 2. Expression of Snail and Twist in HT-29 MCTS. Snail expression in the viable rim (Panel A) and necrotic core (Panel B). Panel C and D show expression of Twist in HT-29 MCTS. E and F show negative primary antibody controls for Snail and Twist respectively.

3.3 Western Blotting analysis of EMT marker expression

3.3.1 HT-29 MCTS

The expression of E-Cadherin, N-Cadherin and Snail was analysed by Western blot in the necrotic core and viable rim populations of HT-29 MCTS. As seen in Figure 3, full

length Snail expression was only found in the viable rim, while in the necrotic core no expression was detected. This supports the immunohistochemical data which showed that Snail was expressed by many of the cells in the inner half of the viable rim, but only a select few in the necrotic core. Whilst some positive cells were observed in the necrotic core by immunohistochemistry (Figure 2), they may have been undetectable on Western blots due to dilution by Snail negative cells or debris.

Full length N-Cadherin was found to be expressed solely in the necrotic core, no detectable expression was seen in the viable rim (Figure 3). This is entirely consistent with the immunohistochemical results (Figure 1). Similarly, full length E-Cadherin expression showed similar results to the immunohistochemistry in the MCTS sections. While expression was seen in both the viable rim and the necrotic core, the expression within the necrotic core was somewhat reduced compared to the viable rim.

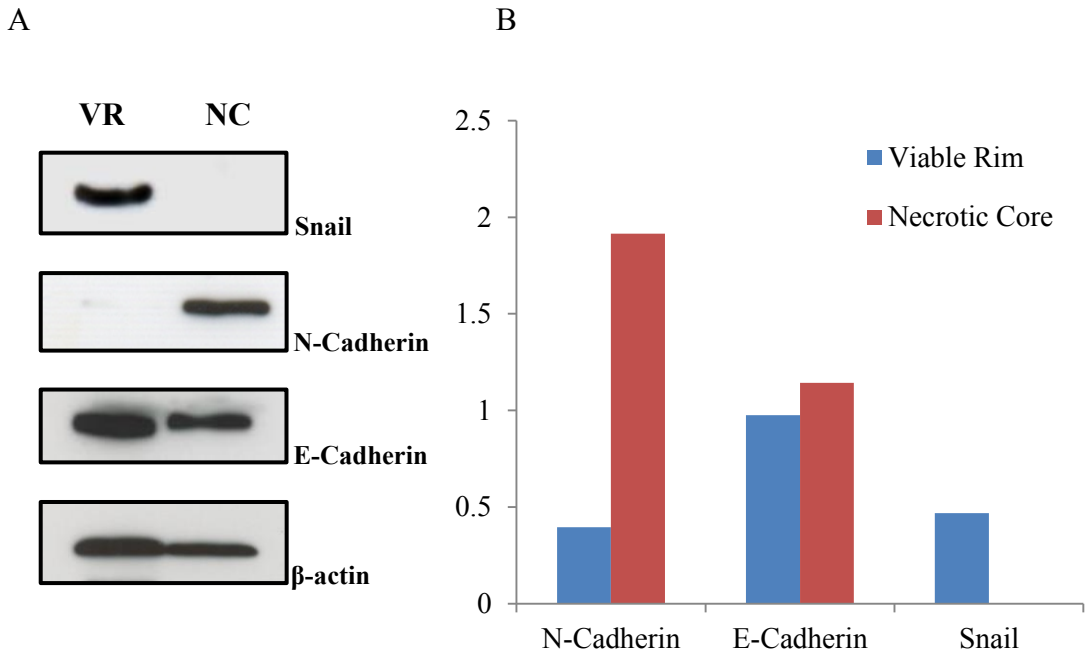


Figure 3 Western Blotting data confirming EMT marker expression within the viable rim and necrotic core of HT-29 MCTS. Panel A shows blots of HT-29 MCTS split into two cell populations, necrotic core and viable rim. Panel B graph displaying the relative expression of the EMT in the different cellular populations, after β-actin normalisation, each experiment was carried out three times.

3.3.2 DLD-1 and HCT-116 MCTS

Western blotting for N-Cadherin was repeated in two separate cell lines, DLD1 and HCT-116, to confirm the results. N-Cadherin was highly expressed within the necrotic core. These results are consistent with the results for HT-29. Both cell lines showed expression of N-Cadherin in the necrotic but no detectable expression within the viable rim.

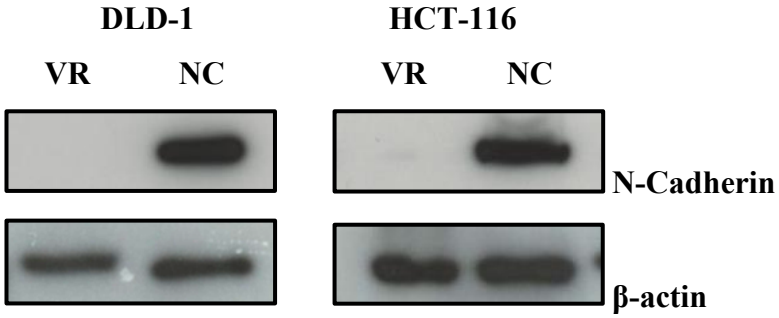


Figure 4. Western Blotting data showing the expression of N-Cadherin in DLD-1 and HCT-116 within the viable rim (VR) and necrotic core (NC) of MCTS.

3.4 Cadherin expression in colorectal xenografts

The qualitative expression of E-Cadherin in a series of human tumour xenografts is presented in Figure 5. The DLD-1 E-Cadherin staining (panel A) was seen to be weaker than that of HT-29, with faint expression seen in only a small proportion of the cells. In the HT-29 xenograft (panel C) strong expression of E-Cadherin was observed in a large number of cells, consistent with the western blotting results for the HT-29 cell line grown as MCTS. HCT 116 xenograft also showed some positive expression of E-Cadherin (panel B), though this xenograft was less strongly stained than HT-29. Faint N-Cadherin expression was seen in all 3 of the xenografts with the strongest expression seen in the HCT 116 xenograft (panel E). With the exception of the staining seen in the HCT 116 cell line, the expression of N-Cadherin was not as wide spread as E-Cadherin and was seen only in small clusters of cells rather than uniformly expressed.

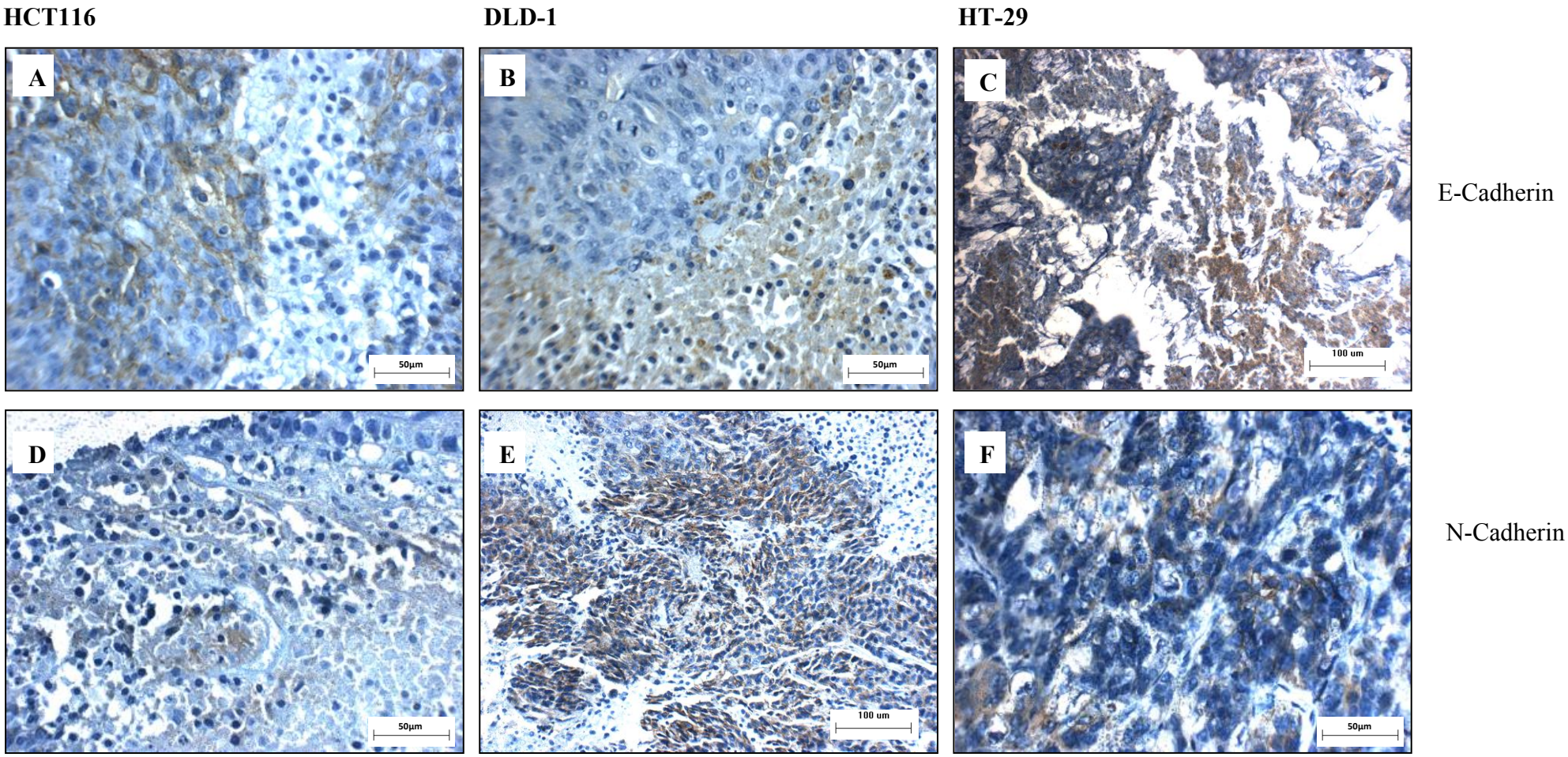


Figure 5 In vivo N-Cadherin and E-Cadherin expression. E-Cadherin expression in (A) DLD-1 (B) HCT 116 and (C) HT-29 xenografts. N-Cadherin expression in (D) DLD-1 (E) HCT 116 and (F) HT-29 xenografts.

3.5 Snail expression in colorectal xenografts

To determine whether the expression of Snail in necrotic cells was confined only to cells grown in spheroids or whether it was a common occurrence in colorectal cancer, three cell lines were grown as xenografts and examined immunohistochemically for Snail expression. Faint expression of Snail was seen in a number of viable cells in the DLD-1 xenograft (Figure 6, panel A). HCT 116 xenograft was completely negative for Snail expression (Figure 6, panel B). In contrast, the HT-29 xenograft showed positive expression of Snail in areas of necrosis (Figure 6, panel C).

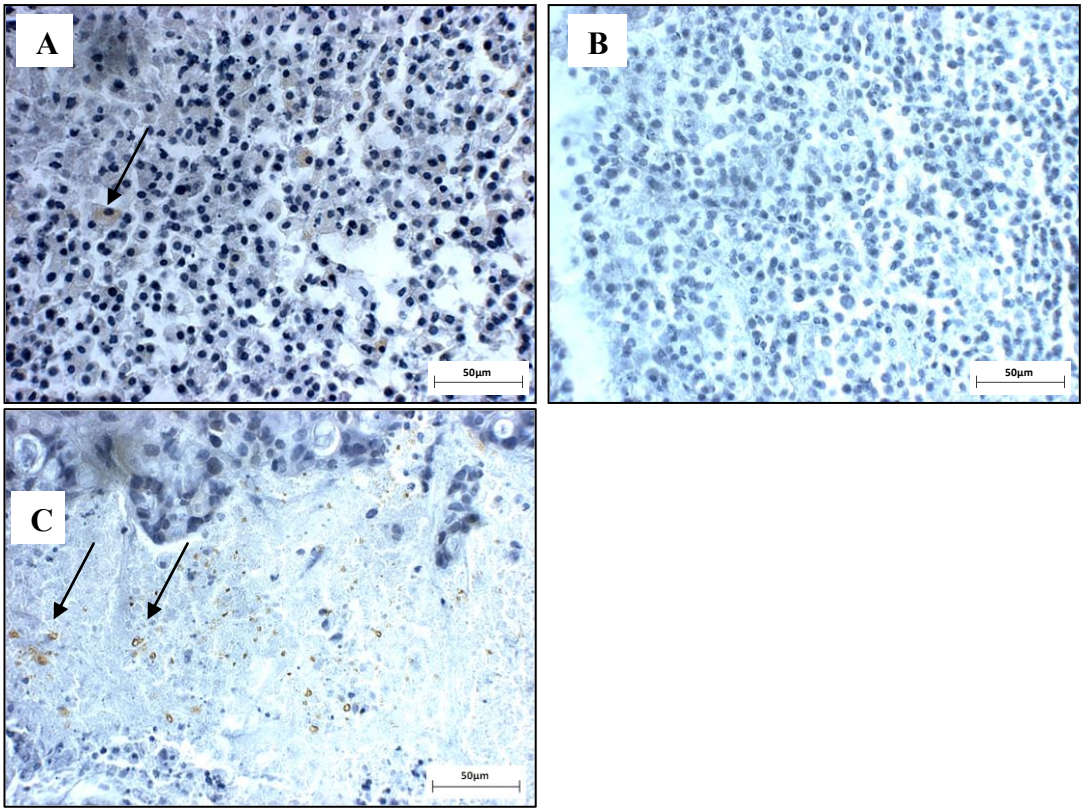


Figure 6. Immunohistochemical analysis of Snail expression in xenografts. Panel A DLD-1, panel B HCT 116 and panel C HT-29, staining in brown.

3.6 CD133, CD44 and CD24 staining to identify stem cell markers within the Necrotic Core.

Stem cells are known to be resistant to anoikis therefore to determine whether the cleaved caspase-3 negative cells in the necrotic core had stem cell characteristics, the expression of a number of known colorectal cancer stem cell markers was examined.

CD133 was found to be expressed in the cytoplasm of most cells throughout the viable rim and necrotic core (Figure 7, panel A). Cytoplasmic expression of CD24 was seen only in a small number of cells within the necrotic core (panel B), while the viable rim was negatively stained. A similar pattern was seen in the CD44 expression (panel C), a small number of cells within the necrotic core were positively stained whilst the rest of the cells in the MCTS were negatively stained.

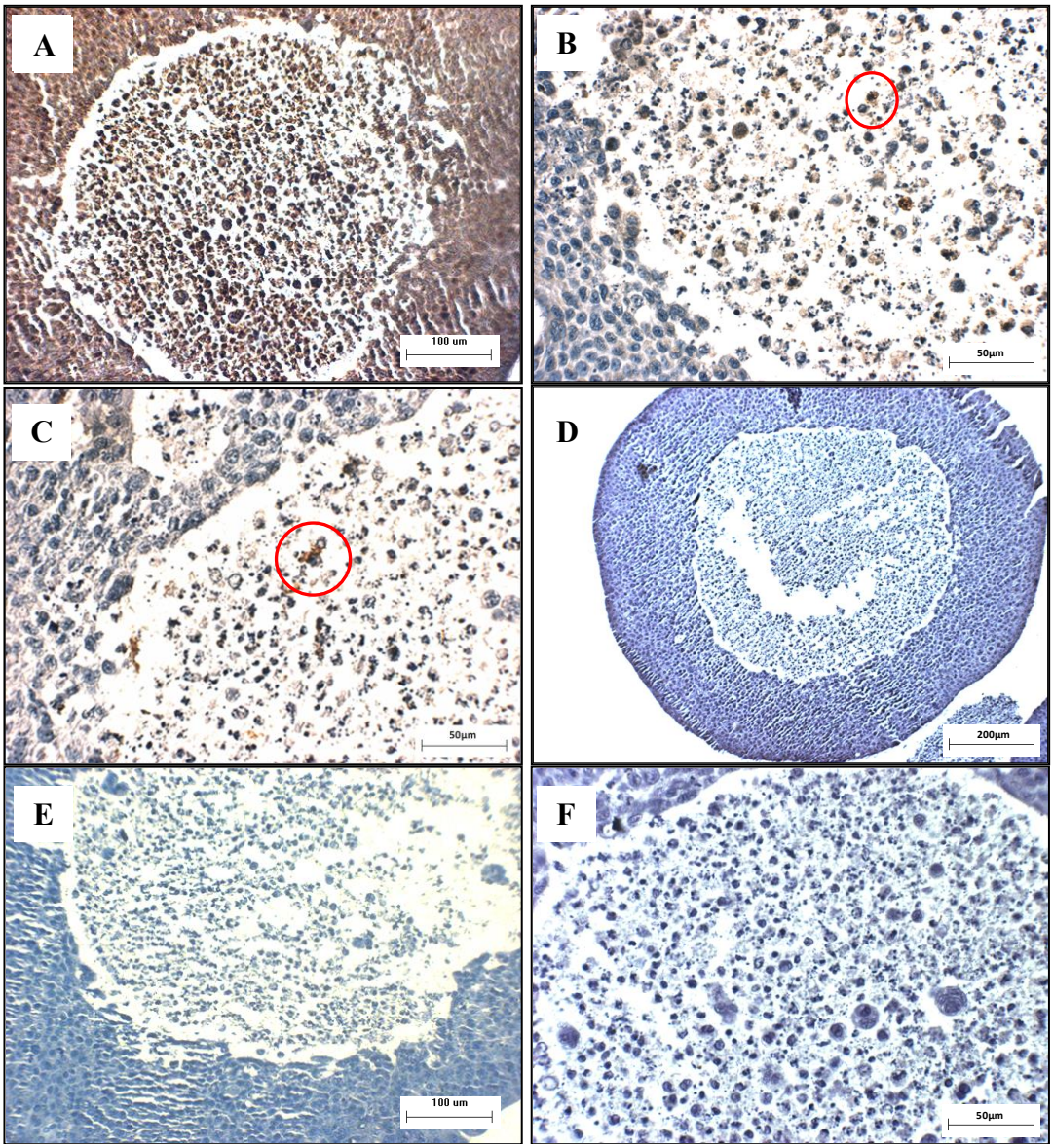


Figure 7 Expression of cell surface receptors' CD133, CD24 and CD44 within HT-29 MCTS. Panel A shows expression of CD133 within the viable rim and necrotic core, Panel B shows CD24 expression in a number of single cells within the necrotic core. Panel C shows expression of CD44 in a cluster of cells in the necrotic core. Panel D, E and F show a negative primary antibody controls for CD133, CD24 and CD44 respectively.

3.7 Sphere Formation Assay to measure anchorage independent growth of Necrotic Core Clones

To measure the anchorage dependent growth of the clones, the sphere-formation assay, which is a functional stem cell assay, was performed. Necrotic Core Clones 1, 2 and 4 all had similar sphere formation capability compared to that of the parental cell line. Two of the clones NCC3 and NCC5 were found to have increased sphere formation abilities. Of these two, NCC5 was found to be significantly higher than that of the HT-29 cells, with a p-value of 0.035.

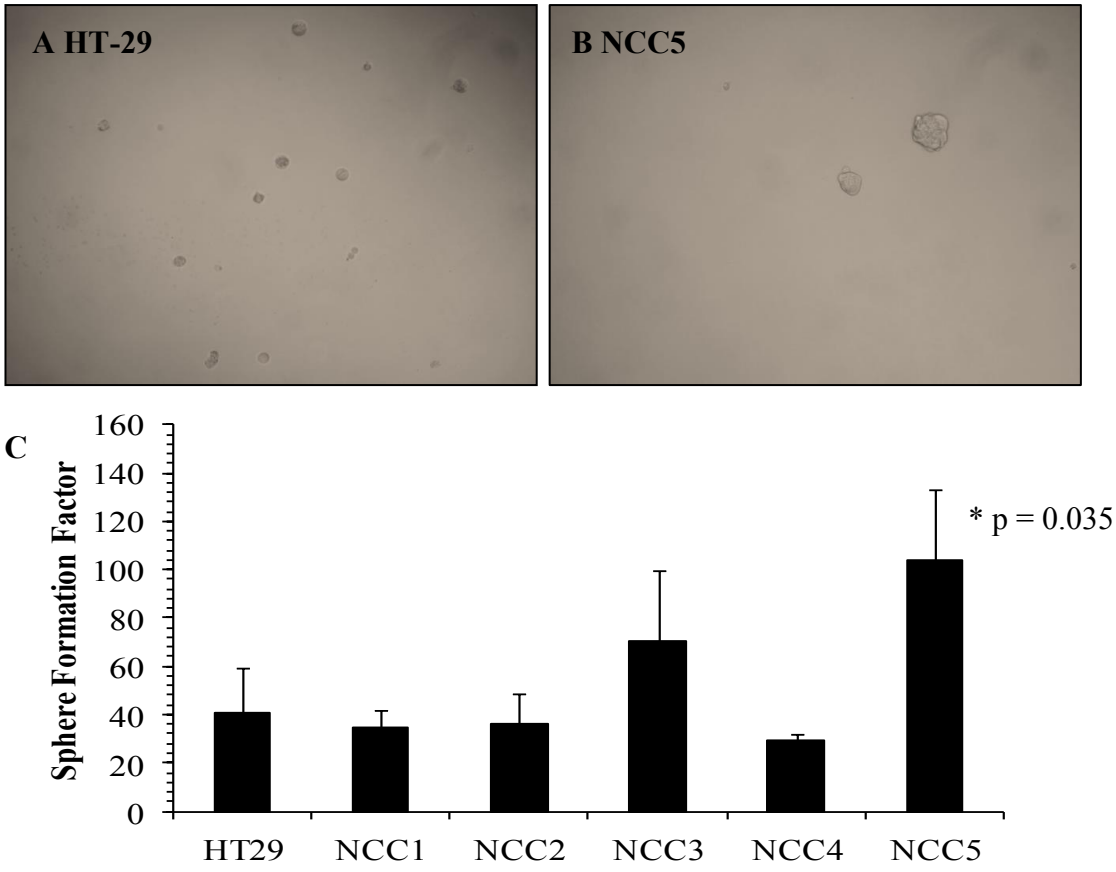


Figure 8. Showing the growth of HT-29 and Necrotic Core Clone 5 in a non-adherent environment in the sphere formation assay. Panel A shows the spheres formed in HT-29 parental cell line, Panel B shows the NCC5 cells. Panel C graph depicting the sphere formation factors for the NCCs and parental cell line calculated from the number and size of the spheres formed per well. Statistical differences between clones and parental cell line were determined using a Student's T Test. Statistical significant differences are denoted with an asterix ($p<0.05$). Error bars were included on the bar to represent the standard error associated with that cell line.

3.8 Wound healing assay to measure the migratory behaviour of Necrotic Core Clones

To assay the migratory phenotype of the necrotic core clones, a wound-healing assay was carried out. This assays measures the rate of 'healing' of a scratch to a monolayer and qualitative and quantitative analysis of results are presented in Figure 9 and Figure 10 respectively. Visually, Necrotic Core Clones 2, 3, 4 and 5 were all shown to be more migratory than the parental cell line (Figure 9). In particular Necrotic Core Clones 2 and 4 were found in some cases to completely close the scratch within 48 hours (see Figure 9) whereas after 48 hours the parental cell line had closed less than 50% of the scratch. Previously completed proliferation assays had showed the clones to be slower growing than the parental cell line (Chapter 1, Table 5), hence the increased ability of the clones to heal the wound is not due to an increased ability to repopulate the wound by proliferation but due to increased migratory behaviour.

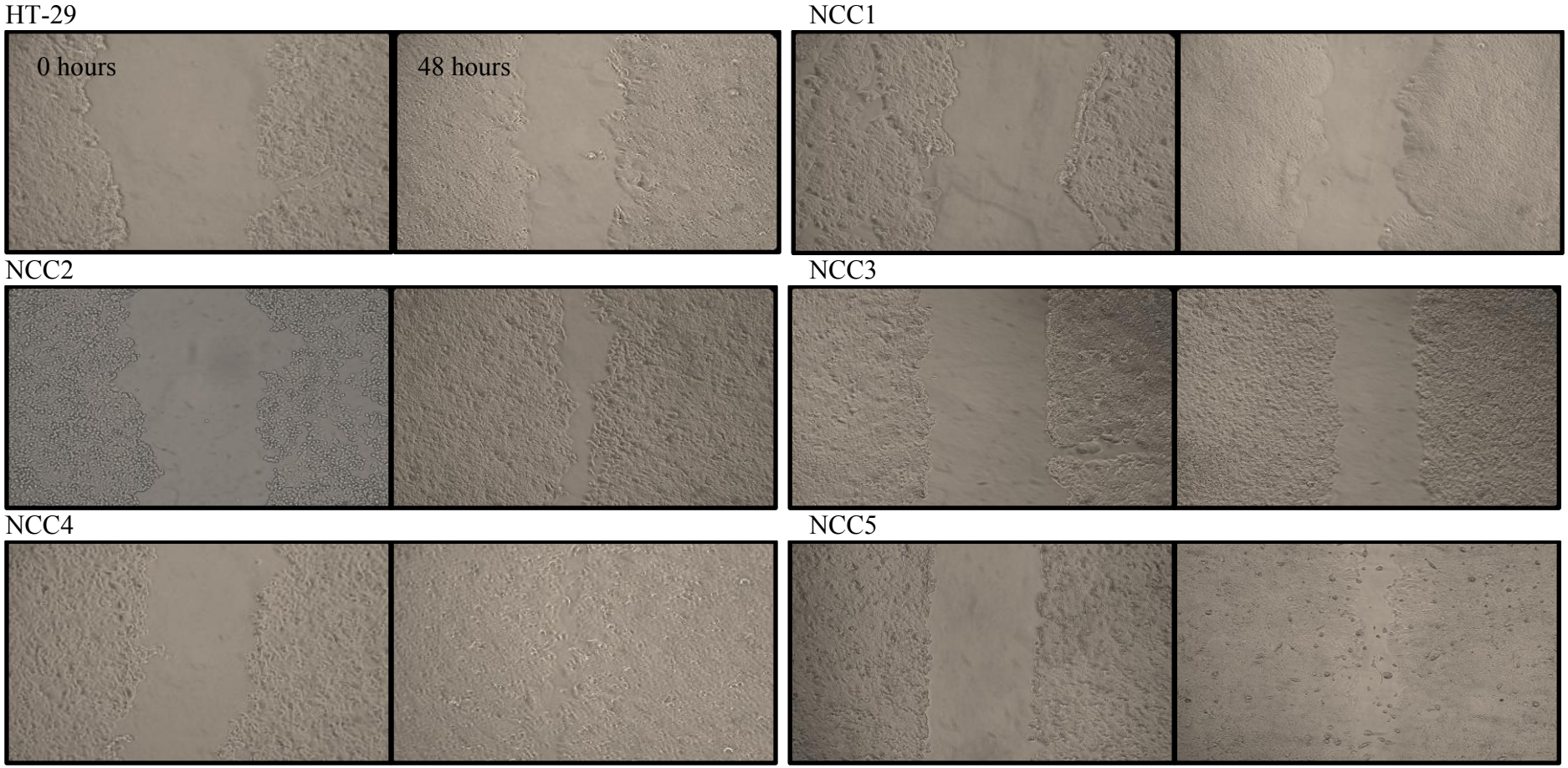


Figure 9. The wound healing assay. Pictures showing the wound made to Parental HT-29 and Necrotic Core Clone (NCC) monolayers at time 0 hours (left) and after 48 hours to allow migration of cells (right).

To ensure that the increases seen in the necrotic core clones was not the result of innate differences in the migratory behaviour of individual cells making up the HT-29 cell line, control clones derived from HT-29 monolayer cultures were generated using the same method as described in Chapter 1, section 2.16, and tested in the wound healing assay. The control clones (CC), while having a range of different migratory behaviours were not significantly more migratory than the HT-29 parental cell line (see Figure 10). When looking at the results statistically using a student's t-test it was found that only Necrotic Core Clone 2 and 4 had significantly different migratory behaviour from the parental cell line.

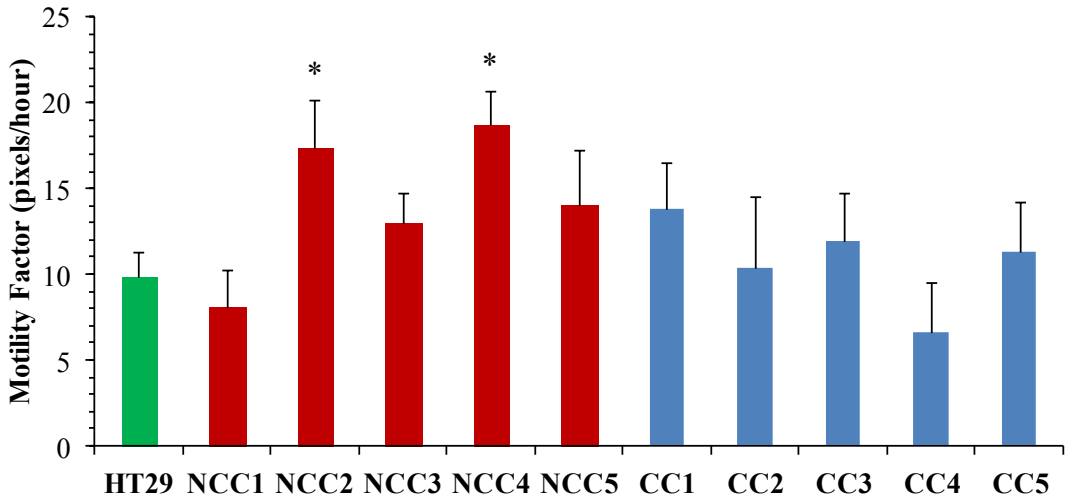
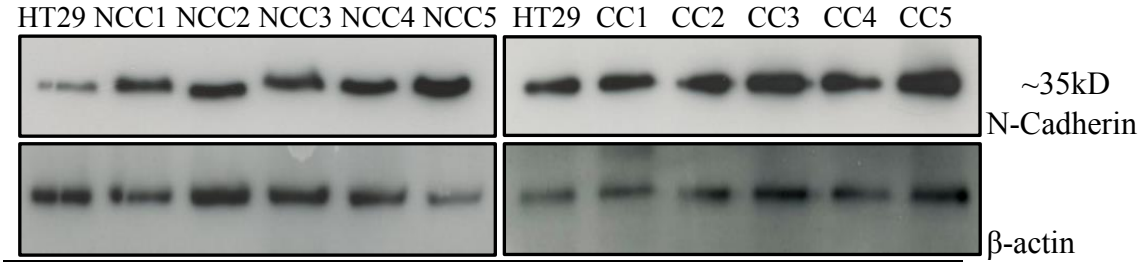


Figure 10. Graph showing the motility of the necrotic core clones, control clones and parental cell line. Scratch widths were measured in 3 separate areas of the scratch and an average derived. Asterix denotes statistically significant difference found through a two tailed Student's T test ($p<0.05$). Both NCC2 and NCC4 were found to have significantly increased motility as shown by p-values of 0.0076 and 0.0064 respectively. Error bars denote standard errors associated with the individual cell lines.

3.9 N-Cadherin Western Blotting Analysis of Clones

To see if the increase seen in the expression of N-Cadherin in the necrotic core of MCTS was transient or stable and if the differing motilities measured in the wound healing assay were linked to EMT status the expression of N-Cadherin was analysed in

the necrotic core clones. While a band of 140kDa was seen in the spheroid fraction, in the clones a band corresponding to a cleaved fragment of N-Cadherin approximately 35kDa was seen instead. This cleaved fragment was seen present at higher levels in all the necrotic core clones compared to the parental cell line. To confirm these differences were not down to the heterogenic nature of the cell line, control clones were analysed. The expression of cleaved N-Cadherin seen in the control clones was more similar to that of the parental cell line than the necrotic core clones, suggesting the necrotic core clones stably express N-Cadherin at a higher level than the parental cell line.



Cell Line	N-Cadherin Band Density	β-Actin Band Density	Normalised N-Cadherin Band Density
HT29	10077	7491	1.35
NCC1	17480	7060	2.48
NCC2	19094	8225	2.32
NCC3	20490	8061	2.54
NCC4	20013	7073	2.83
NCC5	22301	6264	3.56
HT-29	10933	9712	1.13
CC1	12019	9214	1.30
CC2	13229	11411	1.16
CC3	15397	11009	1.40
CC4	13499	10946	1.39
CC5	18500	11818	2.01

Figure 11. N-Cadherin Western Blotting Analysis. Panel A Western Blots of NCC and CCs probed with N-Cadherin antibody (top) and β-Actin antibody (bottom). Panel B Table of Band density data for the western blots, N-Cadherin band density figures are the average of three separate blots. Normalised N-Cadherin ban densities have been normalised using the β-actin band densities

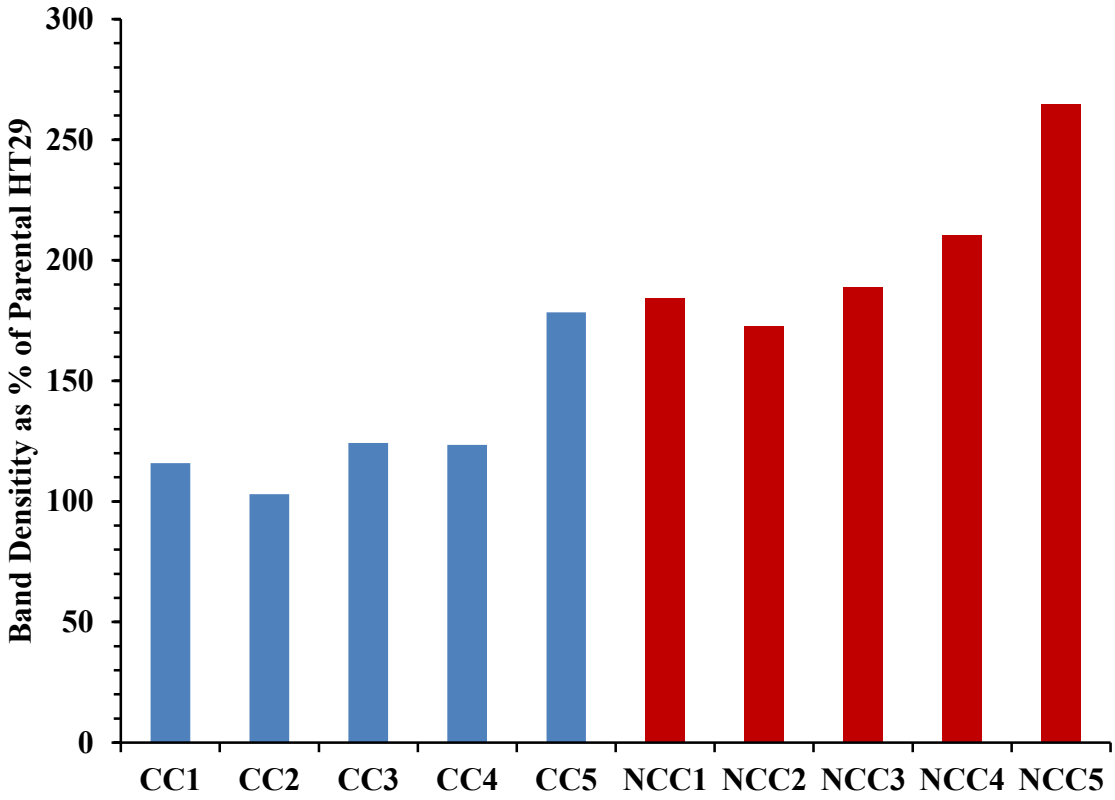


Figure 12. Compilation of N-Cadherin western blotting results to show the differing levels of normalised cleaved N-Cadherin expression in necrotic core and control clones compared to the parental cell line levels.

3.10 In Vivo Growth of HT-29 and Necrotic Core Clone 4

On the basis that NCC4 showed more aggressive, invasive behaviour in the wound healing assay, this cell line was chosen to determine whether it was capable of growing as a solid tumour *in vivo* and its growth characteristics compared to the parental cell line. To compare the tumour forming capabilities of the clones compared to the parental cell line, NCC4 and the parental cell line were injected subcutaneously into the flanks of mice and the results are presented in Figure 13, 14 and 15.

In the HT-29 parental cell line group the take rate was calculated as the number of xenografts reaching palpable size expressed as a percentage of the initial number of inoculations. As all 6 xenografts initially took, the take rate was 100%. Only 2 xenografts however, both within the same mouse, continued to grow for the full

experiment. The other mice were euthanized on days 20 and 22 as the xenografts began to ulcerate. The remaining two xenografts continued to grow until they were removed for histology. The largest grew to 771mm^3 and the smallest to 320mm^3, giving an average tumour volume after 50 days of growth of 546mm^3.

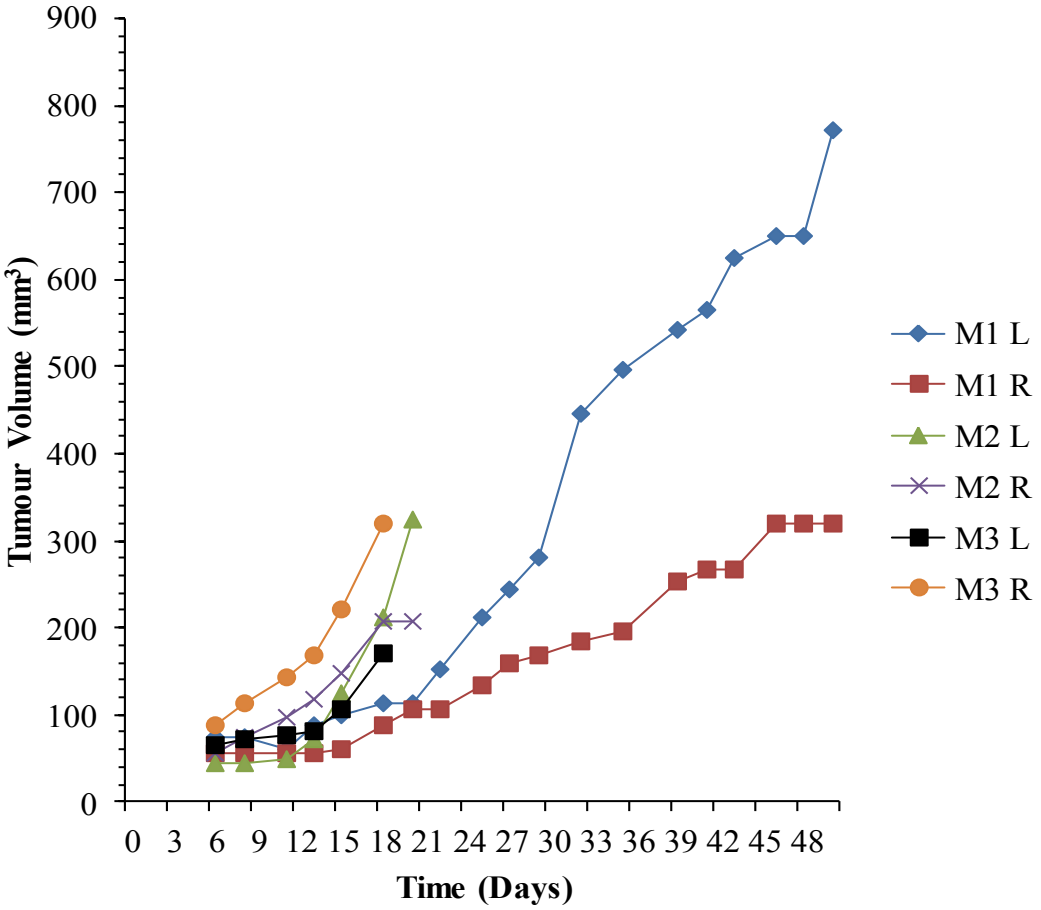


Figure 13. *In vivo* growth curve for HT-29 parental cell line in 3 mice each bearing one xenograft on each flank (L left, R right). All 6 xenografts initially were seen to grow, giving a take rate of 100%. Where the data points end, this represents the point at which the mice were euthanized due to the tumours becoming ulcerated.

In the Necrotic Core Clone 4 group, all 6 xenografts initially took and started to grow giving a take rate of 100%. However the growth of 4 of the 6 xenografts stalled and in some cases, tumour regression occurred, the unsuccessful NCC4 xenografts stopped growing before they reached sizes any bigger than 100mm^3.The two xenografts that

continued to grow for the entire experiment reached sizes of 634 and 446 mm^3, giving an average volume of 540mm^3.

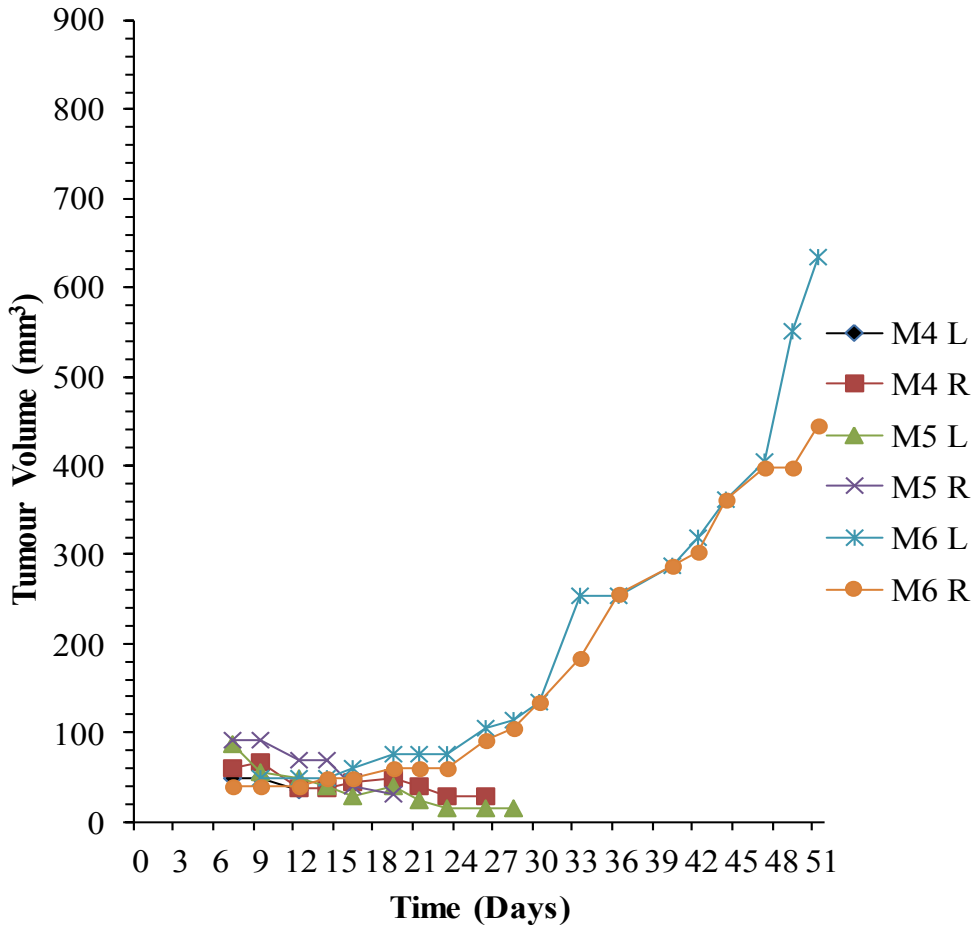


Figure 14. *In vivo* growth curve for Necrotic Core Clone 4 in 3 mice (M4-6) each bearing one xenograft on each flank (L left, R right).

In both cases of the three mice used for each group only one mouse developed tumours which grew successfully. Each mouse developed two tumours, with one growing larger than the other in both cases. Whilst the average tumour size was very similar for both groups the sample size of one for each group means it is difficult to conclude with any confidence whether the similarities seen are true or not. Importantly however NCC4 cells were shown to be capable of forming a xenograft. This cell population was cloned from a colony formed from one single cell and therefore is homogeneous and yet was

able to form a xenograft similar in size to the heterogeneous parental cell line (Figure 15).

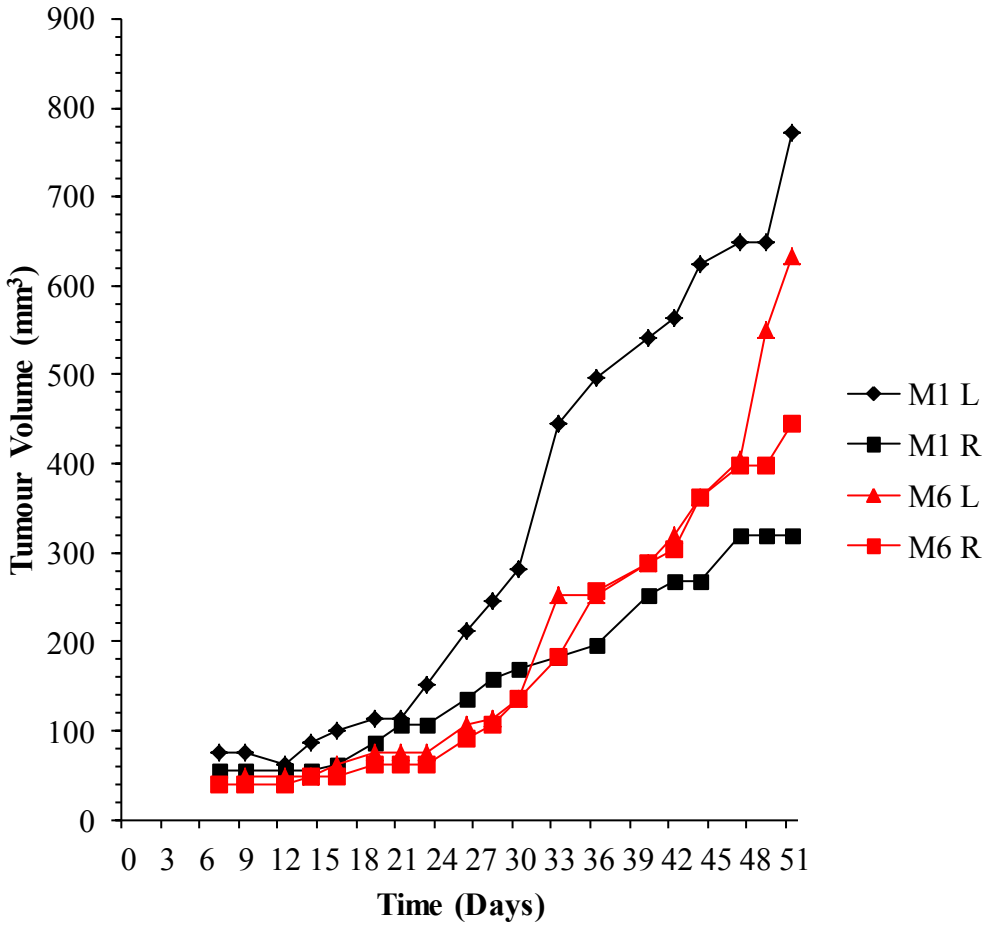


Figure 15. Direct comparison of the growth rates of HT-29 and NCC4 xenografts. M1 represents the HT-29 xenografts that successfully took and continued to grow for the entire experiment, M6 represents the successful NCC4 xenografts. (L left flank, R right flank).

3.11 Immunohistochemical Analysis of HT-29 and Necrotic Core Clone 4 Xenografts

Following the growth of the HT-29 and NCC4 xenografts in the mice, the tumours where excised on day 50, fixed and embedded in preparation for morphological and immunohistochemical analysis. The two xenografts were compared to see if there were any differences between the two tumours formed, morphologically or in terms of marker expression. Markers examined included EMT markers E-Cadherin, N-Cadherin

and Snail, senescence marker p-H2AX, and cell death marker Cleaved Capsase-3. The results showed that xenografts formed from necrotic core clone 4 cells had similar tissue morphology to the parental cell line HT-29, representative images of which are presented in Figure 16, panels A and B. This included extensive areas of necrotic tissue surrounded by healthy tissue. Cleaved Caspase-3 staining demonstrated that apoptosis was occurring in necrotic regions and in areas bordering necrotic regions in both parental and NCC4 xenografts (see Figure 16, panels C and D for representative examples). In contrast to MCTS where cleaved caspase 3 expression was more or less uniform across the necrotic core (see figure 8 in Chapter 1), in the xenografts the staining was less consistent. Whilst necrotic regions were similarly stained in the HT-29 parental xenograft (Figure 16, Panel C) the NCC4 xenografts had regions of strong positive staining typically lining the outer edges of the necrotic regions but not throughout (Figure 16, panel D).

H2AX expression was also examined to see whether there would be differential expression in the necrotic core clone NCC4. In the HT-29 xenograft expression was located in the cells on the periphery of the necrotic regions and in the necrotic regions themselves (Figure 16, panels E and F). This was similar to the pattern of expression seen with the HT-29 cells grown as spheroids.

To determine whether the expression of EMT markers seen in the Necrotic Core of HT-29 MCTS was maintained in the clones the xenografts were examined. Snail expression was seen in necrotic regions of both the HT-29 and NCC4 xenografts (Figure 16, panels G and H) therefore making Snail expression a characteristic of HT-29 necrotic cells when grown *in vitro* and *in vivo*. E-Cadherin expression was seen in both the HT-29 and NCC4 xenografts, and was found to be expressed by a large number of both necrotic and non necrotic cells. Faint N-Cadherin expression was also seen in both xenografts.

The expression of N-Cadherin was not as wide spread as E-Cadherin and was seen only in small clusters of cells rather than uniformly expressed, though membranous staining was seen both in necrotic and necrotic areas of the tissue.

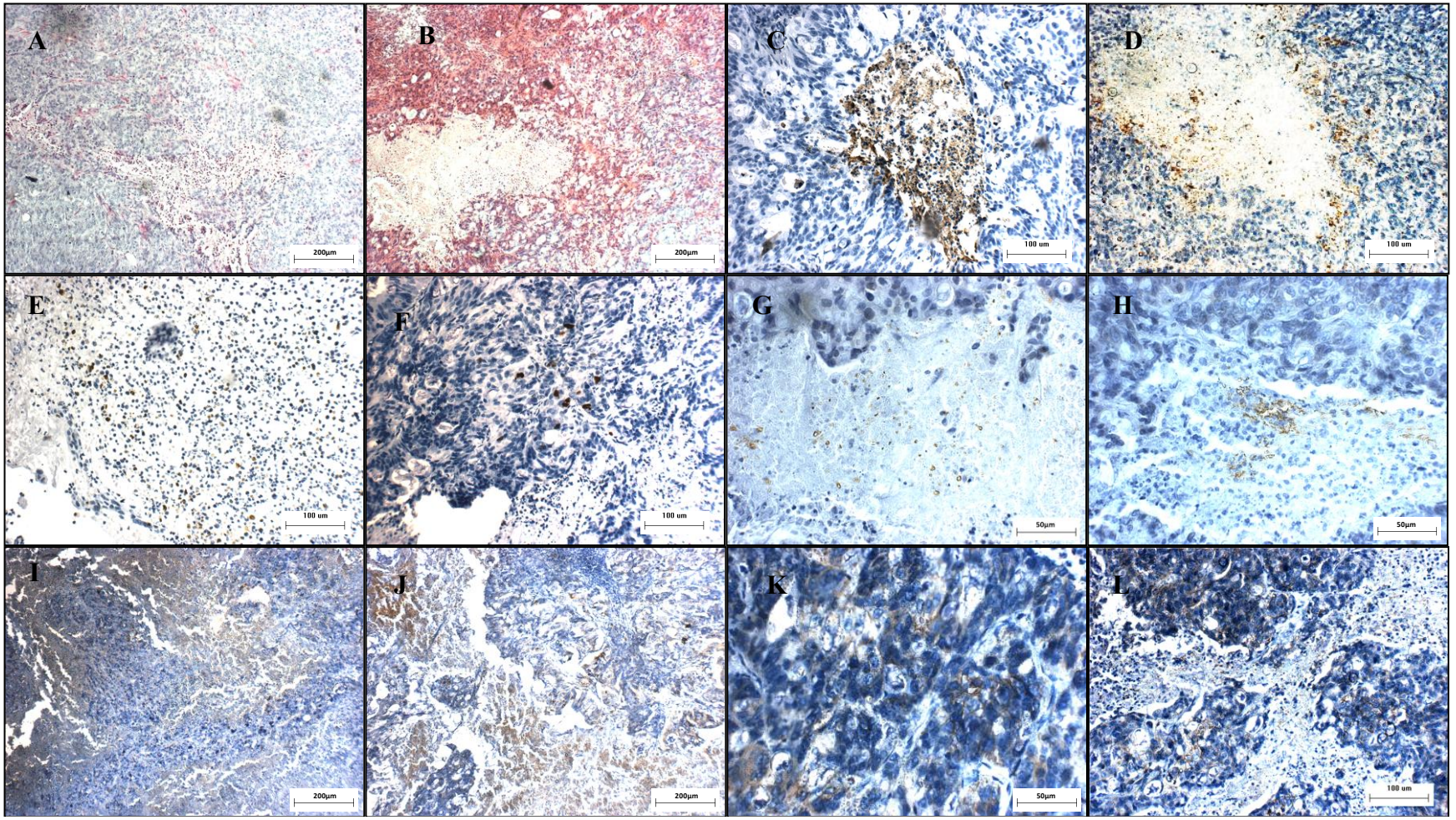


Figure 16. Immunohistochemical analysis of the HT-29 Parental and NCC4 cells grown as xenografts. Analysis of sections stained with Haematoxylin and Eosin (A) HT-29, (B) NCC4. Cleaved caspase-3 expression (C) HT-29, (D) NCC4. H2AX expression (E) HT-29, (F) NCC4. Snail expression (G) HT-29, (H) NCC4. E-Cadherin expression (I) HT-29, (J) NCC4. N-Cadherin expression (K) HT-29, (L) NCC4.

4 Discussion

The aim of Chapter 2 was to characterise the necrotic core clones in order to determine their biological significance. By investigating the possible mechanisms by which they survived the necrotic core and by determining any unique differences between the clones and the parental cell line, new targets may be uncovered. Both the Epithelial Mesenchymal Transition and stem cells have been implicated in the necrotic core clones survival, the evidence for and significance of it is discussed in the following section.

4.1 Surviving the Necrotic Core: Epithelial Mesenchymal Transition

Further to surviving the poor nutrient conditions in the necrotic core the physical environment also places another obstacle for the cells to overcome. The tissue structure within the necrotic core is not very organised, the cells exist in a liquid like state due to the large number of dead cells and cellular debris. The lack of correct cell-cell contacts would normally induce anoikis in epithelial cells, when an epithelial cell becomes unattached from its local environment it undergoes anchorage dependant cell death.[13] Therefore the cells which manage to survive in the necrotic core must have found some way to resist the process of anoikis.

Cells which are capable of bypassing anoikis tend to be those which have undergone the EMT.[14] Hence the necrotic core was investigated for markers of the EMT. What was found in the necrotic core when stained for certain EMT makers was that some cells were seen to have reduced E-Cadherin expression and gain of N-Cadherin expression, this occurred in a number of clusters of cells in the necrotic core only (Figure 1). Loss of E-Cadherin and gain of N-Cadherin are the classical signs of the EMT.[15] Western blots wwere carried out to confirm the immunohistochemistry results, and similar evidence was seen (Figure 3) N-Cadherin was found to be present in the necrotic core

but not in the viable rim population of HT-29 cells. As frequently research is carried out on intact MCTSs, it does not distinguish between the different populations of cells within the MCTS. This is the first time that induction of N-Cadherin expression has been seen specifically in the centre of MCTS. However gain of N-Cadherin expression has been seen in cultures of lung adenocarinoma plural effusions, when grown as spheres compared to their monolayer counterparts.[16] E-Cadherin was found expressed in both populations but the expression in the necrotic core was reduced in comparison to the viable rim. The expression of E-Cadherin found in the MCTS mirrored xenograft expression data reported in the literature where E-Cadherin was found to be lost in areas of necrosis in colorectal xenografts.[17] Furthermore HT-29 cells have previously been found to exhibit a loss of E-Cadherin expression when grown in 3D.[18]

Further markers such as Snail were also investigated (Figure 2), and this staining gave evidence which supported much of the literature which was that Snail is up regulated by reduced oxygen levels.[19, 20] Snail expression was seen in the hypoxic areas of the MCTS, and in a small number of cells in the necrotic core (Figure 2, Panel B). This is consistent with previous studies in breast cancer which have found that hypoxia induced a partial EMT by increasing the expression of Snail.[21] A similar response was seen in pancreatic cancer cells where hypoxia induced Snail expression through the binding of HIF-1α to the hypoxia response elements of the Snail gene promoter. Both in pancreatic and hepatocellular carcinoma this hypoxia induced increase in Snail was associated with metastasis.[22,23] Snail expression has also been reported in the literature to be up regulated when HT-29 cells are grown in 3D, though no distinction in expression has been made between the separate populations of cells within the MCTS. [24]

Together this data indicate these viable cells within the necrotic core may have gone through the EMT. And that this transition may be a key survival mechanism as it helps the cells survive the anchorage independent growth conditions within the necrotic core. To confirm that this was not an isolated occurrence and that it was characteristic of this particular cell line, two other colorectal cancer cell lines were used to form MCTS. Both HCT-116 ($p53^{+}/^{+}$) and DLD-1 MCTS showed a similar pattern of N-Cadherin expression as shown in Figure 4. No expression was seen in the viable rim but it was present in the necrotic core (see Figure 4). This gain of N-Cadherin expression by the cells in the necrotic core is therefore not specific to HT-29 cells and seems to be a common mechanism of colorectal cells grown as MCTS. This phenomenon has also been described in lung adenocarcinoma with similar results reported when monolayer were compared to spheroidal cultures. While cells grown in monolayers were found to be E-Cadherin positive and N-Cadherin negative, the opposite was seen in cells grown in MCTS.[25] However descriptions of colorectal MCTS and their Cadherin expression have yet to contain information of the location of staining within the MCTS instead of treating the MCTS as one population.

Tumour xenografts were examined for Cadherin and Snail expression to see if the results seen in the 3D cell culture mimicked the processes happening *in vivo*. HT-29 and DLD-1 xenografts were both seen to have Snail expression within areas of necrotic tissue whilst HCT-116 was not (Figure 6). N-Cadherin expression was found throughout all the xenografts including in areas of necrotic tissue (Figure 5).

Together this evidence shows that the necrotic core of MCTS induces a full or partial EMT in epithelial cancer cells, and this acquisition gives the cancer cells an increasingly metastatic phenotype. Furthermore the changes in expression of EMT markers are

mirrored in an *in vivo* setting which supports the use of MCTS to study this phenomenon.

4.2 EMT in the Necrotic Core Clones

Once the necrotic core clones had been established in cell culture their metastatic potential and EMT status was characterised. This would establish whether the changes seen in the necrotic core of the MCTS were retained by the cells once they returned to favourable growth conditions. Since living cells have not previously been studied from the necrotic core of spheroids their growth characteristics and properties represent novel findings.

One of the first observations made about the clones when they were returned to normal monolayer growth conditions in cell culture was that they continued to grow in suspension forming small MCTS. The cells maintained their preferences for growth in suspension for several passages before returning to fully adherent growth. Spontaneous spheroid formation, a characteristic of the EMT,[26,27] was not seen in the original cell line under the same culture conditions suggesting that the shift from epithelial to mesenchymal phenotype seen in the necrotic core is maintained in the clones.

Through wound healing assays, it was clear that some of the clones under normal culture conditions had a higher migratory ability than the parental cell line. The expression levels of N-Cadherin, an EMT marker found elevated in the necrotic core of MCTS, was examined in clones via western blotting and all five of the clones had higher levels of the cleaved form of N-Cadherin than the parental cell line. This experiment, performed on monolayer cultures of the clones, determined whether the clones maintained the increased levels of N-Cadherin seen in MCTS or whether this increase was transient. Clearly the results showed that this effect was not transient.

While the full length N-Cadherin protein was not present in the cells grown as monolayer a shortened part of the protein corresponding to the intracellular and transmembrane domains was seen. This cleaved form of N-Cadherin has been reported previously to be found upregulated in gliobastoma cells and was shown to be cleaved by the disintegrin and metalloproteinase ADAM10. Whilst the function of the cleaved form of N-Cadherin has not been conclusively established, in Glioblastoma when ADAM 10 cleavage of N-Cadherin was inhibited a decrease in cell migration was seen. [28]
While an increased migratory ability is not ideal when treating cancer, unless the cell can anchor itself into a new environment and use its self renewal properties to form a secondary tumour, this ability will not result in successful metastasis. Increased migratory behaviour and the ability to grow in suspension, two properties the clones have, supports the view that they have undergone the EMT. Generally anchorage dependant cells such as epithelial cells will not survive if they become unattached from their surrounding, this lack of cellular attachment leads to anoikis. The ability of the cells to survive within the 'liquid like' environment of the MCTS necrotic core in the first place suggests that these cells must have undergone the EMT. But having undergone the EMT does not ensure that once the cells have migrated to a new site they are capable of forming a metastatic deposit. Recent research into the mode of growth of tumours has put forth the hypothesis that stem cells are the seed of tumour growth and it is these cancer associated stem cells that drive the proliferation and differentiation of tumours. According to the hypothesis migratory stem cells which have an EMT-like phenotype are responsible for metastasis.[29]

4.3 The Necrotic Core as a Niche for Stem-like cells

Other markers known to be up regulated in three dimensional cell cultures are stem cell markers. Stem cell markers are known to be up-regulated in cells exposed to hostile microenvironments such as those found at the centre of MCTS.[30] Stem cells themselves are known to have increased survival capabilities compared to cancer cells, both in response to chemotherapeutics and poor nutrient and oxygen conditions. Hypoxic niches have been known to exist which support colorectal cancer stem cells and hypoxia as a stimuli has been discovered to enrich stem cell populations.[31,32] Furthermore the EMT marker Snail which was found to be expressed in hypoxic and necrotic regions within the MCTS and xenografts has been linked to the acquisition of a stem cell phenotype. [33, 34]

Because the identification of cancer stem cells can be complicated, several assays were used to determine the 'stemness' of cells. While normal tissue stem cells are often identified by the expression of certain stem cell markers, this is not such a reliable method when looking at cancer stem cells due to their intrinsic plasticity.

Three known colorectal cancer stem cells markers, CD133, CD24, and CD44 were examined in MCTS and suggested that the viable cells in the necrotic core were expressing stem cell markers. While CD133 was found to be expressed by the majority of the cells within the HT-29 MCTS population, cytoplasmic expression of CD24 and CD44 was only found in a small number of cells within the necrotic core. All three stem cell markers examined have been previously found to be expressed in subpopulations of cells within the HT-29 cell line.[35, 36] Specifically cytoplasmic staining for CD44 and CD24 expression has also been found in HT-29 populations.[37, 38, 39] Furthermore none of the cells within the necrotic core were actively proliferating as seen by the absence of Ki-67 expression (Chapter 1, section 3.4), which is consistent with cancer stem cells

which are known not to rapidly proliferate and tend to exist in a quiescent state.[40] The expression of the two stem cell markers suggested that these viable cells in the necrotic core could be cancer stem cell, or had acquired stem-like characteristics.

To confirm whether the necrotic core clones were also stem-like in their behaviour, functional assays were also carried out in order to back up the marker expression data. The clones' anchorage independent growth was measured in the sphere formation assay which found that one of the clones, Necrotic Core Clone 5 did had a significantly greater ability to form spheres than the parental cell line (Figure 8). The final functional assay that was carried out was the xenograft assay. The *in vivo* growth of Necrotic Core Clone 4 and the parental cell line were measured, and it was determined that the clone was fully capable of forming a heterogeneous xenograft similar to that formed by the parental cell line. The NCC4 xenograft managed to recapitulate both the morphology and marker expression of the HT-29 parental cell line. The only difference between the two was the expression of cleaved capsase-3, the levels of which was reduced in the clone and differences in localisation were seen. The lack of staining within the necrotic areas could mean that the clone cells are more resistant to cell death or that when grown in vivo these cells can be subject to caspase independent cell death mechanisms. Altogether the expression of certain stem cell markers found on a subset of cells within the necrotic core, along with the evidence for EMT having occurred and the in vivo growth assays show these migratory cells have an ability to form tumours *in vivo* that recapitulate the morphology of the original cell line, and hence should be investigated further.

4.4 Clone Summary

Overall the clones have different phenotypes than the parental cell line in many different ways. They have been shown to be more migratory than the parental cell line in the wound healing assay (Figure 9) and they possess the capabilities for anchorage independent growth as determined by the sphere formation assay (Figure 8). In addition, the clones retain the ability to form heterogeneous tumours *in vivo* with growth characteristics that are similar to the original parental lines. These observations together demonstrate that there are viable cells within the necrotic core that can regrow when conditions become favourable, have a greater capacity to migrate and are able to form tumours when implanted subcutaneously into mice. Certain cancer stem cell makers were found on cells within the necrotic core of the MCTS, though the expression of stem cell markers exhibits a certain amount of plasticity and is known to be dependent upon the cells' current microenvironment. This links into the experimental conditions surrounding the formation of the clones. Whilst it was found that necrotic core cells could and did return to proliferation once placed in standard culture conditions, this does not necessarily mean that if necrotic core cells *in vivo* or in a clinical setting were once again re-introduced to sufficient oxygen and nutrient, the same phenomenon would be seen. Henceforth future works into the subject would benefit from looking into whether necrotic core cells could re-grow a tumour *in vivo* when transplanted directly into mice without returning to normal cell culture conditions first.

Whether the necrotic core of MCTS acts to enrich these cell populations or whether harsh microenvironmental factors such as severe hypoxia cause a change within the cell is not yet known. However the fact remains that the necrotic core of spheroids provides a niche for cancer stem cell-like cells which have a phenotype consistent with metastatic cells. Furthermore that this differential phenotype, as compared to the parental cell line,

is maintained when the cells are returned to normal culture conditions showing that the changes selected for in the necrotic core are not transient. The targeting of these cells therefore is crucial to prevent disease recurrence.

Regardless of whether they are called stem cells, stem-like cell or cancer initiating cells, these cells have shown themselves to be more migratory and capable of self renewal. Therefore not considering their title, these cells seem likely candidates for disease recurrence and are therefore biologically significant.

5 References

[1] Mohammadizadeh F, Ghasemibasir H, Rajabi P, Naimi A, Eftekhari A, Mesbah A. Correlation of E-cadherin expression and routine immunohistochemistry panel in breast invasive ductal carcinoma. *Cancer Biomark*. 2009;5(1):1-8.

[2] Kim MA, Lee HS, Lee HE, Kim JH, Yang HK, Kim WH. Prognostic importance of epithelial-mesenchymal transition-related protein expression in gastric carcinoma. *Histopathology*. 2009;54(4):442-51.

[3] Shi Y1, Wu H, Zhang M, Ding L, Meng F, Fan X. Expression of the epithelial-mesenchymal transition-related proteins and their clinical significance in lung adenocarcinoma. *Diagn Pathol*. 2013;8:89.

[4] Dayem AA, Choi HY, Kim JH, Cho SG. Role of oxidative stress in stem, cancer, and cancer stem cells. *Cancers (Basel)*. 2010;2(2):859-84

[5] Kruyt FA, Schuringa JJ. Apoptosis and cancer stem cells: Implications for apoptosis targeted therapy. *Biochem Pharmacol*. 2010;80(4):423-30.

[6] Chen K, Huang YH, Chen JL. Understanding and targeting cancer stem cells: therapeutic implications and challenges. *Acta Pharmacol Sin*. 2013;34(6):732-40.

[7] Beauséjour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P, Campisi J. Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J*. 2003;22(16):4212-22.

[8] La Porta CA, Zapperi S, Sethna JP. Senescent cells in growing tumors: population dynamics and cancer stem cells. *PLoS Comput Biol*. 2012;8(1):e1002316.

[9] Nautiyal J, Kanwar SS, Yu Y, Majumdar AP. Combination of dasatinib and curcumin eliminates chemo-resistant colon cancer cells. J Mol Signal. 2011;6:7-30.

[10] Domínguez D, Montserrat-Sentís B, Virgós-Soler A, Guaita S, Grueso J, Porta M, Puig I, Baulida J, Francí C, García de Herreros A. Phosphorylation regulates the subcellular location and activity of the snail transcriptional repressor. *Mol Cell Biol*. 2003;23(14):5078-89.

[11] Luo WR, Li SY, Cai LM, Yao KT. High expression of nuclear Snail, but not cytoplasmic staining, predicts poor survival in nasopharyngeal carcinoma. *Ann Surg Oncol*. 2012;19(9):2971-9.

[12] Muenst S, Däster S, Obermann EC, Droeser RA, Weber WP, von Holzen U, Gao F, Viehl C, Oertli D, Soysal SD. Nuclear expression of snail is an independent negative prognostic factor in human breast cancer. *Dis Markers*. 2013;35(5):337-44.

[13] Liotta LA, Kohn E. Anoikis: cancer and the homeless cell. *Nature*. 2004;430(7003):973-4.

[14] Guadamillas MC, Cerezo A, Del Pozo MA. Overcoming anoikis--pathways to anchorage-independent growth in cancer. *J Cell Sci*. 2011;124(Pt 19):3189-97.

[15] Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol*. 2014;15(3):178-96.

[16] Giarnieri E, De Vitis C, Noto A, Roscilli G, Salerno G, Mariotta S, Ricci A, Bruno P, Russo G, Laurenzi A, Giovagnoli MR, Ciliberto G, Mancini R. EMT markers in lung adenocarcinoma pleural effusion spheroid cells. *J Cell Physiol*. 2013;228(8):1720-6.

[17] Simiantonaki N, Kurzik-Dumke U, Karyofylli G, Jayasinghe C, Kirkpatrick CJ. Loss of E-cadherin in the vicinity of necrosis in colorectal carcinomas: association with NFkappaB expression. *Int J Oncol.* 2007;31(2):269-75.

[18] Han XY, Wei B, Fang JF, Zhang S, Zhang FC, Zhang HB, Lan TY, Lu HQ, Wei HB. Epithelial-mesenchymal transition associates with maintenance of stemness in spheroid-derived stem-like colon cancer cells. *PLoS One*. 2013;8(9):e73341.

[19] Zhang L, Huang G, Li X, Zhang Y, Jiang Y, Shen J, Liu J, Wang Q, Zhu J, Feng X, Dong J, Qian C. Hypoxia induces epithelial-mesenchymal transition via activation of SNAI1 by hypoxia-inducible factor -1α in hepatocellular carcinoma. *BMC Cancer*. 2013;13:108.

[20] Imai T, Horiuchi A, Wang C, Oka K, Ohira S, Nikaido T, Konishi I. Hypoxia attenuates the expression of E-cadherin via up-regulation of SNAIL in ovarian carcinoma cells. *Am J Pathol*. 2003;163(4):1437-47.

[21] Lundgren K, Nordenskjöld B, Landberg G. Hypoxia, Snail and incomplete epithelial-mesenchymal transition in breast cancer. *Br J Cancer*. 2009;101(10):1769-81.

[22] Zhang L, Huang G, Li X, Zhang Y, Jiang Y, Shen J, Liu J, Wang Q, Zhu J, Feng X, Dong J, Qian C. Hypoxia induces epithelial-mesenchymal transition via activation of SNAI1 by hypoxia-inducible factor -1α in hepatocellular carcinoma. *BMC Cancer*. 2013;13:108.

[23] Zhu GH, Huang C, Feng ZZ, Lv XH, Qiu ZJ. Hypoxia-induced snail expression through transcriptional regulation by HIF-1α in pancreatic cancer cells. *Dig Dis Sci*. 2013;58(12):3503-15.

[24] Han XY, Wei B, Fang JF, Zhang S, Zhang FC, Zhang HB, Lan TY, Lu HQ, Wei HB. Epithelial-mesenchymal transition associates with maintenance of stemness in spheroid-derived stem-like colon cancer cells. *PLoS One*. 2013;8(9):e73341.

[25] Giarnieri E, De Vitis C, Noto A, Roscilli G, Salerno G, Mariotta S, Ricci A, Bruno P, Russo G, Laurenzi A, Giovagnoli MR, Ciliberto G, Mancini R. EMT markers in lung adenocarcinoma pleural effusion spheroid cells. *J Cell Physiol.* 2013;228(8):1720-6.

[26] Pease JC, Brewer M, Tirnauer JS. Spontaneous spheroid budding from monolayers: a potential contribution to ovarian cancer dissemination. *Biol Open*. 2012;1(7):622-8.

[27] Xu MH, Gao X, Luo D, Zhou XD, Xiong W, Liu GX. EMT and acquisition of stem cell-like properties are involved in spontaneous formation of tumorigenic hybrids between lung cancer and bone marrow-derived mesenchymal stem cells. *PLoS One*. 2014;9(2):e87893.

[28] Kohutek ZA, diPierro CG, Redpath GT, Hussaini IM. ADAM-10-mediated N-cadherin cleavage is protein kinase C-alpha dependent and promotes glioblastoma cell migration. *J Neurosci*. 2009;29(14):4605-15.

[29] Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat Rev Cancer*. 2005;5(9):744-9.

[30] Taddei ML, Giannoni E, Comito G, Chiarugi P. Microenvironment and tumor cell plasticity: an easy way out. *Cancer Lett*. 2013;341(1):80-96.

[31] Mao Q, Zhang Y, Fu X, Xue J, Guo W, Meng M, Zhou Z, Mo X, Lu Y. A tumor hypoxic niche protects human colon cancer stem cells from chemotherapy. *J Cancer Res Clin Oncol*. 2013;139(2):211-22.

[32] Das B, Tsuchida R, Malkin D, Koren G, Baruchel S, Yeger H. Hypoxia enhances tumor stemness by increasing the invasive and tumorigenic side population fraction. *Stem Cells*. 2008;26(7):1818-30.

[33] Hwang WL, Yang MH, Tsai ML, Lan HY, Su SH, Chang SC, Teng HW, Yang SH, Lan YT, Chiou SH, Wang HW. SNAIL regulates interleukin-8 expression, stem cell-like activity, and tumorigenicity of human colorectal carcinoma cells. *Gastroenterology*. 2011 Jul;141(1):279-91, 291.e1-5.

[34] Zhou W, Lv R, Qi W, Wu D, Xu Y, Liu W, Mou Y, Wang L. Snail contributes to the maintenance of stem cell-like phenotype cells in human pancreatic cancer. *PLoS One*. 2014;9(1):e87409.

[35] Hwang WL, Yang MH, Tsai ML, Lan HY, Su SH, Chang SC, Teng HW, Yang SH, Lan YT, Chiou SH, Wang HW. SNAIL regulates interleukin-8 expression, stem cell-like activity, and tumorigenicity of human colorectal carcinoma cells. *Gastroenterology*. 2011 Jul;141(1):279-91, 291.e1-5.

[36] Elsaba TM, Martinez-Pomares L, Robins AR, Crook S, Seth R, Jackson D, McCart A, Silver AR, Tomlinson IP, Ilyas M. The stem cell marker CD133 associates with enhanced colony formation and cell motility in colorectal cancer. *PLoS One*. 2010;5(5):e10714.

[37] Weichert W, Denkert C, Burkhardt M, Gansukh T, Bellach J, Altevogt P, Dietel M, Kristiansen G. Cytoplasmic CD24 expression in colorectal cancer independently correlates with shortened patient survival. *Clin Cancer Res*. 2005;11(18):6574-81.

[38] Weichert W, Denkert C, Burkhardt M, Gansukh T, Bellach J, Altevogt P, Dietel M, Kristiansen G. Cytoplasmic CD24 expression in colorectal cancer independently correlates with shortened patient survival. *Clin Cancer Res*. 2005;11(18):6574-81.

[39] Ke J, Wu X, Wu X, He X, Lian L, Zou Y, He X, Wang H, Luo Y, Wang L, Lan P. A subpopulation of $CD24^{+}$ cells in colon cancer cell lines possess stem cell characteristics. *Neoplasma*. 2012;59(3):282-8.

[40] Li L, Bhatia R. Stem cell quiescence. *Clin Cancer Res.* 2011;17(15):4936-41.

Chapter 3:Therapeutic Implications

1 Introduction

Chemotherapy is one of the main methods used to treat cancer. However development of resistance is a major cause of treatment failure in cancer patients. Resistance to both conventional chemotherapeutics and more specific targeted therapies share many common mechanisms including alterations in target, increased efflux of drug, inactivation of cell death signalling and up-regulation in pro survival signalling. Survival of resistant cells following treatment can lead to relapse as the resistant cells repopulate the tumour resulting in a tumour which is now resistant to treatment.[1] The existence of viable cells within necrotic regions which are not only less sensitive to standard chemotherapeutics due to their non proliferative status but maintain this increased resistance once returned to proliferation could well explain the link between tumour necrosis and poor prognosis. Furthermore stem cells are known to be more resistant to chemotherapeutics through various different mechanisms including increased expression of multidrug transporters,[2] and the utilisation of autophagy.[3, 4] The aim of this chapter is the determine the therapeutic responses of the clones to several commonly used chemotherapeutics to determine if they are significantly more resistant compared to the parental cell line. This research will be carried out using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay, which utilises the conversion of formazan crystals from MTT by living cells to determine the number of living cells. This can be used to measure the effects of cytotoxic drugs upon cells.

2 Methods

2.1 Chemosensitivity testing using the MTT Assay

The chemosensitivity of the Necrotic Core Clones and the parental HT-29 cell line was examined using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Initially cells were seeded in round bottom wells of a 96 well plate at the seeding density of $2x10^3$ cells per well. Once cells were seeded into wells, they were left to adhere for 24 hours at 37^oC 5% CO_2 and 95% humidity. One lane on each plate contained no cells only McCoy's 5a Modified Medium to serve as the blank, and another lane contained cells but no drug, the serve as the control. After 24 hours cells were treated with increasing concentrations of Doxorubicin (Sigma Aldrich, St Louis, MO, USA, stock solution 10mM, dissolved in H_2O), 5-Fluorouracil (Sigma Aldrich, St Louis, MO, USA, stock solution 100mM, dissolved in DMSO) and Gefitinib (Selleckchem, TX, USA, stock solution 100mM, dissolved in DMSO). The cells were incubated with the drug for 96 hours. Following this incubation period, 20µl of MTT (5mg/ml) was added to each well and left for a further 4 hours. All media containing MTT was then removed, leaving the resulting formazan crystals in the bottom of the wells. The crystals were then dissolved in 150µl of DMSO (Dimethyl sulfoxide, Sigma, St Louis, MO, USA) before absorbance was read using a plate reader at 540nm. Percent survival was calculated with the help of the control drug lane (cells but no drug) as formation of the formazan product correlates with cell number. The following formula was used to calculate percent survival:

$$\%\, Survial = \left(\frac{absorbance\ of\ treated\ cells}{absorbance\ of\ control\ cells}\right) \times 100$$

3 Results

3.1 Chemosensitivities of clones derived from the necrotic core.

Once clones from the necrotic core had been established and were growing as monolayers the response of cells to chemotherapeutic drugs was determined. Three different drugs used to treat colorectal cancer in the clinic were tested (doxorubicin, 5-fluorouracil (5FU) and gefitinib) and the response of the parental HT-29 and the necrotic core clones to each of these drugs is presented below. IC_{50} values, which represent the concentration of drug required to kill 50% of the cell population, for all drugs are summarised in Table 4.

3.2 Response of NCC and HT-29 cells to doxorubicin

Using doxorubicin, the IC_{50} value for the clones were all similar to the parental cell line with the exception of Necrotic Core Clone 1. NCC1 was found to be significantly more resistant to doxorubicin than HT-29 cells, with IC_{50} values of 144 ± 39μM and 70 ± 17μM for NCC1 and HT-29 respectively (Figure 1). In contrast, NCC4 was significantly ($p < 0.05$) more sensitive than the parental HT-29 with IC_{50} values of 36 ± 9 and 70± 17 μM respectively (figure 1).

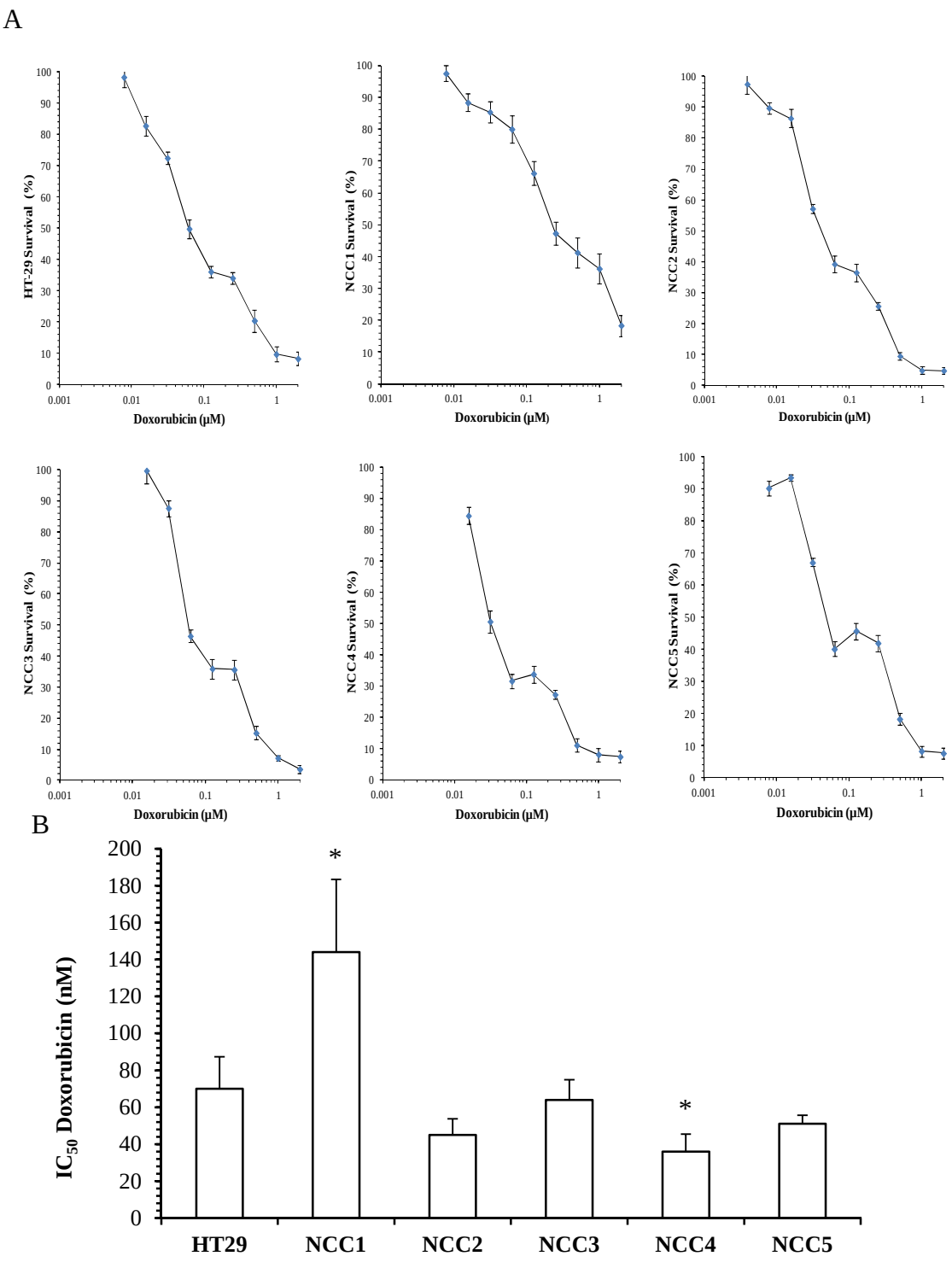


Figure 1. Doxorubicin Dose Response Curves. Panel A shows the dose response curves for the HT-29 parental cells and Necrotic Core Clones in response to doxorubicin. Panel B presents the IC_{50} values for each of the different cells. All experiments were carried out in triplicate, error bars denote the standard deviations associated with the data. Clones which have statistically significant ($p<0.05$) IC_{50} values compared to the parental cell line are marked with an asterix.

When statistically analysed Necrotic Core Clone 1 was found to be significantly more resistant to Doxorubicin than the parental cell line with a p-value of 0.04 which was below the significance threshold of 0.05 (Table 1). Necrotic Core Clone 4 was found to be significantly more sensitive to Doxorubicin than the parental cell line with a p-value of 0.04.

Table 1. Results of a 2-Tailed Student's T-Test. Calculating the statistical significance of the clones' chemosensitivities compared to the parental cell line in the case of Doxorubicin. Significance as measured by a p-value of <0.05 denoted by *).

Cell Line	P-value
NCC1	0.040338*
NCC2	0.093746
NCC3	0.658858
NCC4	0.043251*
NCC5	0.152285

3.3 Response of NCC and HT-29 cells to 5-FU

Another chemotherapeutic agent used in the treatment of colorectal cancer is 5-FU. When the chemosensitivities of the clones to 5-FU was compared to the parental cell line a similar result to the doxorubicin chemosensitivity was seen. Again Necrotic Core Clone 1 was found to have a significantly decreased sensitivity to the drug. In this case exhibiting an IC_{50} almost three times greater compared to HT-29 (Figure 2). This difference in IC_{50} was statistically significant ($p < 0.01$, Table 2). In contrast, NCC3 was more sensitive to 5FU than HT-29 and this difference was statistically significant ($p < 0.05$). The rest of the clones all had statistically comparable IC_{50} values to the parental cell line, despite the fact that the IC_{50} value for NCC5 was the lowest value observed ($p > 0.05$, Table 2).

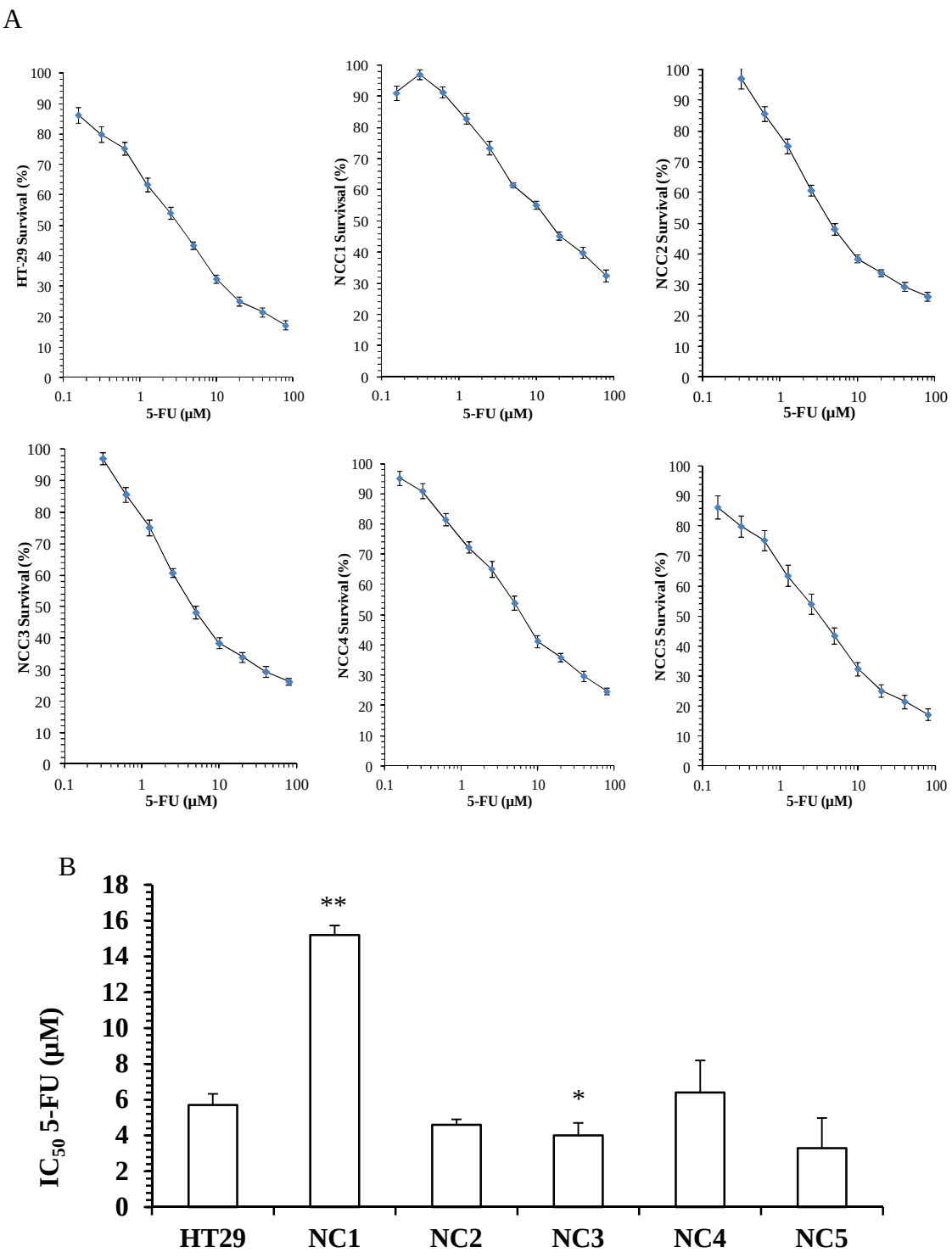


Figure 2. Response of necrotic core clones and the parental HT-29 cell line to 5-FU. Panel A shows Dose Response Curves for all 5 clones and parental cell line. Panel B shows the calculated IC_{50} values. Cells were exposed to 5-FU for 96 hours and each value represents the mean ± standard deviation for three independent experiments. Clones which have statistically significant ($p<0.05$*, $p<0.001$**) IC50 values compared to the parental cell line are marked with an asterix.

Table 2. Results of a 2-Tailed Student's T-Test. Calculating the statistical significance of the clones' 5-FU IC_{50} compared to the parental cell line. (Significance as measured by a p-value of <0.05 and 0.001 denoted by * and ** respectively).

Cell Line	P-value
NCC1	0.000036**
NCC2	0.057992
NCC3	0.038351*
NCC4	0.523350
NCC5	0.083682

3.4 Response of NCC and HT-29 cells to gefitinib

The response of the clones to gefitinib was determined, the results of which are presented in Figure 3. Whilst some of the NCC lines showed some increased selectivity to gefitinib, there were no statistically significant differences ($p > 0.05$) in IC_{50} values between NCC and parental HT-29 cell (Table 3).

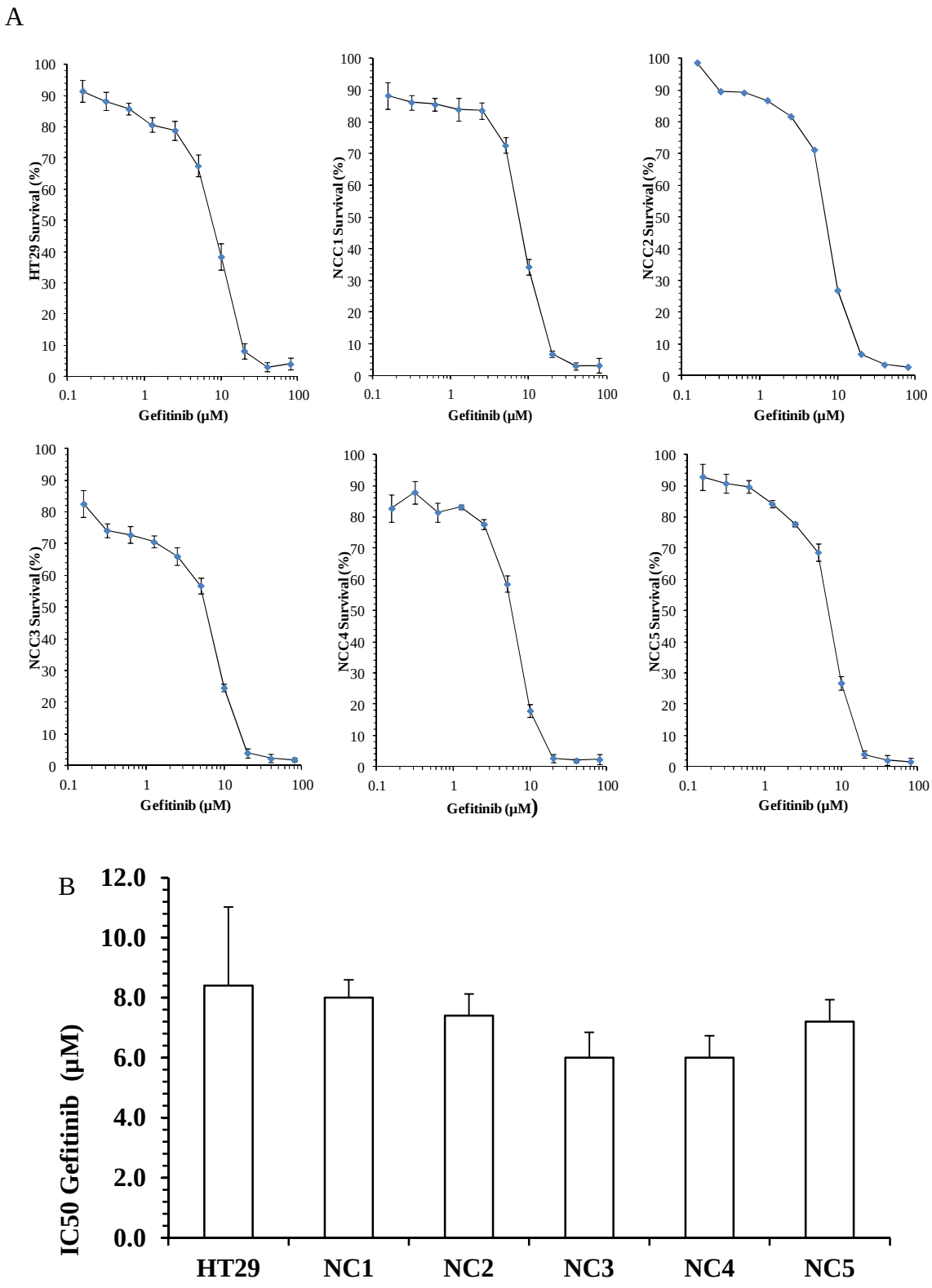


Figure 3. Chemosensitivities to the Necrotic Core Clones and Parental Cell Line to gefitinib. Panel A shows individual dose response curves for each of the clones and the parental cell line detailing cell death in response to concentration of drug. Panel B shows a bar chart presenting the gefitinib IC_{50}s for the necrotic core clones and parental cell line.

When a statistical analysis in the form of a Student's T-Test was carried out no significant differences were found to be present between the clones and the parental cell line (Table 3).

Table 3. **Results of a 2-Tailed Student's T-Test.** Calculating the statistical significance of the clones' Gefitinib IC50 compared to the parental cell line.

Cell Line	P-value
NCC1	0.801437
NCC2	0.551095
NCC3	0.202862
NCC4	0.202322
NCC5	0.489259

3.5 Chemosensitivity Summary

Certain patterns were seen in the response of the clones to chemotherapeutic agents (Table 4 A). In the case of the two drugs which targeted actively proliferating cells (doxorubicin and 5-FU), NCC1 was found to be significantly more resistant compared to the parental cell line. This clone has a resistance mechanism that the parental cell line and other necrotic core clone do not. This resistance mechanism was not of use when the cells were treated with the EGFR inhibitor gefitinib suggesting that the mechanism is independent of this receptor.

In order to try and understand the differences in chemosensitivity of NCC1 to 5-FU and doxorubicin the doubling rates of the clones grown as monolayers (Table 4B, as presented in Chapter 1, Table 4) were examined. NCC1 had the largest doubling time, 30.89 ± 0.36 hours compared to the parental cell line's (25.65 ± 1.91 hours). The reduced rate of cellular proliferation could explain the reduced sensitivity to 5-FU and doxorubicin.

Table 4. A Chemosensitivities of the necrotic core clones and the parental HT-29 cell line. Cells were exposed to each drug for 96 hours and cell survival was determined using the MTT assay. Each value represents the mean ± standard deviation for three independent experiments and significance at p values of < 0.01 and <0.05 are denoted by ** and * respectively). **B Measurement of cellular proliferation of the clones grown as monolayers (Chapter 1, Table 4).** Statistically significant differences between the clones and parental cell line were determined using a Two Tailed Student's T Test, significance (<0.05) is denoted by an asterix.

A

Cell Line	5FU (μM) ± SD	Doxorubicin (nM) ± SD	Gefitinib (μM) ± SD
HT29	5.7 ± 0.6	70 ± 17	8.4 ± 2.6
NCC1	15.2 ± 0.5**	144 ± 39*	8.0 ± 0.6
NCC2	4.6 ± 0.3	45 ± 9	7.4 ± 0.7
NCC3	4.0 ± 0.7*	64 ± 11	6.0 ± 0.8
NCC4	6.4 ± 1.8	36 ± 9*	6.0 ± 0.7
NCC5	3.3 ± 1.7	51 ± 5	7.2 ± 0.7

B

Cell Line	Cell Doubling (hours)
HT-29	25.65 ± 1.91
NCC1	30.89 ± 0.36*
NCC2	30.38 ± 2.02
NCC3	26.99 ± 0.48
NCC4	29.99 ± 0.81*
NCC5	28.43 ± 1.60

4 Discussion

Previous chapters in this thesis have demonstrated that within the necrotic core of MCTS, there are cells that retain viability and these can form new cell lines when returned to favourable growth conditions. Furthermore, these cells can also show increased motility and have gone through the EMT leading to the possibility that these cells could be more aggressive when they re-grow upon their return to favourable conditions. This chapter has focused on whether the cell lines derived from the necrotic core have differential response to chemotherapeutic agents that are typically used to treat colorectal cancer.

Drugs used to treat cancer which are targeted against actively proliferating cells will target cells in the viable rim (of MCTS) and tumour areas closest to blood vessels. Necrotic areas by the virtue of their proliferation status are not affected.[5] This was not considered to be an issue when they were believed to only contain dead cells. However now it seems that some cells can survive in this hostile environment and retain permanent changes this may present a problem.

When looking at the chemosensitivity of the clones, two of the five were seen to react to all three chemotherapeutics in a similar manner to the parental cell line. However one of the clones, NCC1, showed a significantly decreased sensitivity in response to both doxorubicin and 5-floururacil (5-FU). Whilst NCC3 was also significantly more resistant to 5-FU, NCC4 was found to be more sensitive to doxorubicin (Table 4). As both doxorubicin and 5-FU target actively proliferating cells,[6] the proliferation rates of the clones were examined alongside their response to the drugs in order to determine whether rate of proliferation correlated with chemoresistance. NCC1 and NCC4 had significantly decreased rates of proliferation compared with the parental cell line, though only NCC1 was found to have increased resistance to the any of the drugs

tested. Whilst NCC3 did not have significantly altered rate of proliferation compared the parental cell line. From the small number of clones it is difficult to confidently suggest that the rate of cellular proliferation is linked to the cells' sensitivity to the anti proliferative drugs. Though it does seem to be a difference that all the necrotic core clone cell lines share, as all 5 of the clones exhibit slower doubling times than the parental cell line. A slower metabolic/proliferative rate may be a protective mechanism for the cell, there is much information published in the literature regarding the resistance mechanisms for 5-FU and doxorubicin which supports this hypothesis. Slow cycling cancer cells were found to be more resistant to 5-FU in colon carcinoma patients.[7] Cellular proliferation rates in tumours have been linked to clinical response in breast cancer patients.[8] More specifically expression of cell cycle related genes, especially genes involved in G1 to S phase transition were found to be attenuated in 5-FU resistant colorectal and breast cancer cells. The resistant cells were characterised as slower growing with a higher proportion of the cells in GO/G1 and lower proportion in S-phase.[9] Further supporting evidence found in the literature also linked increased doubling time with increased 5-FU resistance in colon cancer cell lines.[10] The mechanism behind this is believed to be explained by attenuated cell cycling resulting in a slower incorporation 5-FU into the cell's DNA thereby giving the cell longer to repair to misincorporated nucelotides.[11]Similar results have been described in response to doxorubicin where fast growing breast cancer cells were shown to be more sensitive than slower growing cell lines,[12] and within cell lines slower growing, stem cell marker positive cells were also found to be more resistant than their faster growing stem cell marker negative counterparts.[13]

Both 5-FU, an anti-metabolite and doxorubicin, a DNA intercalator[14,] have both been found previously to be less effective on cancer stem cells. In the case of 5-FU this

resistance was found to involve Wnt signalling, in $CD133^+$ DLD-1 colorectal cancer stem cells. 5-FU was found to cause an increase in Wnt signalling, whereas in normal DLD-1 cells, caused a decrease in Wnt signalling. Furthermore the resistance seen in the $CD133^+$ cells was reduced by the inhibition of Wnt signalling.[15] Increased chemoresistance is a characteristic of cancer stem cells, as described in Chapter 2 there is evidence to suggest that the clones may have stem like characteristics. Doxorubicin, which also inhibits the activity of topoisomerase II,[16, 17] has also been linked to increased Wnt signalling and over expression of multidrug resistance transporters, both of which are characteristics of cancer stem cells.[18] Together doxorubicin and 5-FU have been used in research to help select for cancer stem cells by taking advantage of the stem cells increased resistance to both drugs.[19]Chemoresistance of cancer stem cells has been given as the reason for tumour recurrence.[20] In fact by treating the tumour with an agent to which cancer stem cells are resistant or less sensitive than the rest of the tumour population could act to enrich the cancer stem cell population within this tumour and therefore leads to recurrence this time of an even more chemoresistant tumour.[21] If indeed cancer stem cells are able to survive within harsh microenvironments and avoid chemotoxicity then new targets must be found in order to eliminate them.

The final conclusion is that whilst some cell lines derived from the necrotic core have a similar chemosensitivity profile to parental lines, others have a more resistant phenotype that could have significant therapeutic implications. Not only may these cells go on to form secondary tumours but due to the conditions within the necrotic core this may contribute towards the evolution of a resistant population of cells. Whether they acquire this increased resistance through mutations gained within the necrotic core or whether the necrotic core selects for them, the end result is a cell which is harder to kill with conventional chemotherapy. Furthermore resistance to the two drugs 5-FU and

doxorubicin is commonly found in stem cells, lending further weight to the argument that the living cells found in the necrotic core of MCTS could be stem-like cells. Therefore by targeting stem cell resistance mechanisms it may be possible to target these cells. This discovery could help explain why high levels of tumour necrosis have been linked to increased tumour aggression. The evolution of an increasingly resistant sub population within areas of necrotic tissue is a hypothesis that could explain this. While it might seem counterintuitive, there have been many scenarios put forward to explain the relationship between necrosis and tumour aggression. For instance highly angiogenic tumours are seen to have higher levels of necrosis and this is due to areas of the highly proliferating tumour constantly outgrowing the angiogenic capacity of the tumour's vasculature leading to necrosis. However while there are theories explaining the relationship between necrosis and aggression none have yet been confirmed. By looking closely at the necrotic core in MCTS and necrotic core clones it may be possible to determine a mechanism for this phenomenon. Though what is certain from the piece of research is that the Necrotic Core Clones have therapeutic significance which could lead to therapeutic implications for the necrotic core.

5 References

[1] Giaccone G, Pinedo HM. Drug Resistance. *Oncologist*. 1996;1(1 & 2):82-87.

[2] Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer*. 2005;5(4):275-84.

[3] Wu S, Wang X, Chen J, Chen Y. Autophagy of cancer stem cells is involved with chemoresistance of colon cancer cells. *Biochem Biophys Res Commun*. 2013;434(4):898-903.

[4] Cufí S, Vazquez-Martin A, Oliveras-Ferraros C, Martin-Castillo B, Vellon L, Menendez JA. Autophagy positively regulates the CD44(+) CD24(-/low) breast cancer stem-like phenotype. Cell Cycle. 2011 Nov 15;10(22):3871-85.

[5] Corrie PG. Cytotoxic chemotherapy: clinical aspects. *Medicine.* 2008; 36 (1): 24–28.

[6] Malhotra V, Perry MC. Classical chemotherapy: mechanisms, toxicities and the therapeutic window. *Cancer Biol Ther*. 2003;2(4 Suppl 1):S2-4.

[7] Sun Q, Zhong Y, Wu F, Zhou C, Wang D, Ma W, Zhang Y, Zhang S. Immunotherapy using slow-cycling tumor cells prolonged overall survival of tumor-bearing mice. *BMC Med*. 2012;10:172.

[8] Amadori D, Volpi A, Maltoni R, Nanni O, Amaducci L, Amadori A, Giunchi DC, Vio A, Saragoni A, Silvestrini R. Cell proliferation as a predictor of response to chemotherapy in metastatic breast cancer: a prospective study. *Breast Cancer Res Treat*. 1997;43(1):7-14.

[9] Wang W, Cassidy J, O'Brien V, Ryan KM, Collie-Duguid E. Mechanistic and predictive profiling of 5-Fluorouracil resistance in human cancer cells. *Cancer Res*. 2004;64(22):8167-76.

[10] Guo X, Goessl E, Jin G, Collie-Duguid ES, Cassidy J, Wang W, O'Brien V. Cell cycle perturbation and acquired 5-fluorouracil chemoresistance. *Anticancer Res*. 2008;28(1A):9-14.

[11] Zhang N, Yin Y, Xu SJ, Chen WS. 5-Fluorouracil: mechanisms of resistance and reversal strategies. *Molecules*. 2008;13(8):1551-69.

[12] Ahmad IM, Mustafa EH, Mustafa NH, Tahtamouni LH, Abdalla MY. 2DG enhances the susceptibility of breast cancer cells to doxorubicin. *Cent Eur J Biol*. 2010;5(6):739-748.

[13] Yenigun VB, Ozpolat B, Kose GT. Response of CD44+/CD24-/low breast cancer stem/progenitor cells to tamoxifen‐ and doxorubicin‐induced autophagy. *Int J Mol Med*. 2013;31(6):1477-83.

[14] Swift LP, Rephaeli A, Nudelman A, Phillips DR, Cutts SM. Doxorubicin-DNA adducts induce a non-topoisomerase II-mediated form of cell death. *Cancer Res*. 2006;66(9):4863-71.

[15] Deng YH, Pu XX, Huang MJ, Xiao J, Zhou JM, Lin TY, Lin EH. 5-Fluorouracil upregulates the activity of Wnt signaling pathway in CD133-positive colon cancer stem-like cells. *Chin J Cancer*. 2010;29(9):810-5.

[16] Thorn CF, Oshiro C, Marsh S, Hernandez-Boussard T, McLeod H, Klein TE, Altman RB. Doxorubicin pathways: pharmacodynamics and adverse effects. *Pharmacogenet Genomics*. 2011;21(7):440-6.

[17] Nitiss JL. Targeting DNA topoisomerase II in cancer chemotherapy. *Nat Rev Cancer*. 2009;9(5):338-50.

[18] Flahaut M, Meier R, Coulon A, Nardou KA, Niggli FK, Martinet D, Beckmann JS, Joseph JM, Mühlethaler-Mottet A, Gross N. The Wnt receptor FZD1 mediates chemoresistance in neuroblastoma through activation of the Wnt/beta-catenin pathway. *Oncogene*. 2009;28(23):2245-56.

[19] Vu NB, Nguyen TT, Tran LC, Do CD, Nguyen BH, Phan NK, Pham PV. Doxorubicin and 5-fluorouracil resistant hepatic cancer cells demonstrate stem-like properties. *Cytotechnology.* 2013;65(4):491-503.

[20] Dick JE. Stem cell concepts renew cancer research. *Blood*. 2008;112(13):4793-807.

[21] Wicha MS, Liu S, Dontu G. Cancer stem cells: an old idea--a paradigm shift. *Cancer Res*. 2006;66(4):1883-90; discussion 1895-6.

Chapter 4: Proteomic analysis of the Necrotic Core Clones

1 Introduction

Whilst the Human Genome Project was completed in 2003, the Human Proteome Project only began in 2010.[1] Genomic investigations in cancer provide huge volumes of information which have lead to many new targets for cancer research. Proteomics however can be argued as being a better tool to understand disease as it looks at the functional unit of the cells. While a genetic change may not lead to any change in function, a proteomic change will often make a functional difference at the cellular level.[2] Investigations into the proteomic profile of cancer cells began over five decades with the discovery of patterns of globulin expression using the newly developed starch gel electrophoresis technology. [3] Since then the development of new technologies have allowed the quantitative analysis of large numbers of proteins within cells. Progress thus far has included the characterisation of multiple types of cancer from tissues, cells and serum with the goal of elucidating signalling pathways and the aberrant expression.

In the previous three chapters it has been demonstrated that the cells cloned from the necrotic core of MCTS are different from the parental cell line and hence may have biological and therapeutic implications. Therefore it is important to develop therapeutic strategies to target these cells. As a first step in the process this chapter examines the proteome of the cells derived from the necrotic core with the aim to identifying any potential targets for drug development.

2 Methods

2.1 Overview

To examine the proteome of the NCCs in comparison to the parental cell line, protein was extracted from the relevant cell pellets and concentrated before being digested with trypsin and labelled with iTRAQ labels. Once the individual samples were labelled they were combined into one sample and separated into 11 elutions using strong cation exchange. The eluate where then applied to the Reverse Phase Nano HPLC to separate them further onto MALDI plates. Following this MALDI TOF/TOF analysis was performed on the mass spectrometer, before the data was transferred to ProteinScape 3.0 for protein identification and further analysed using Gene Ontology software (Figure 1).

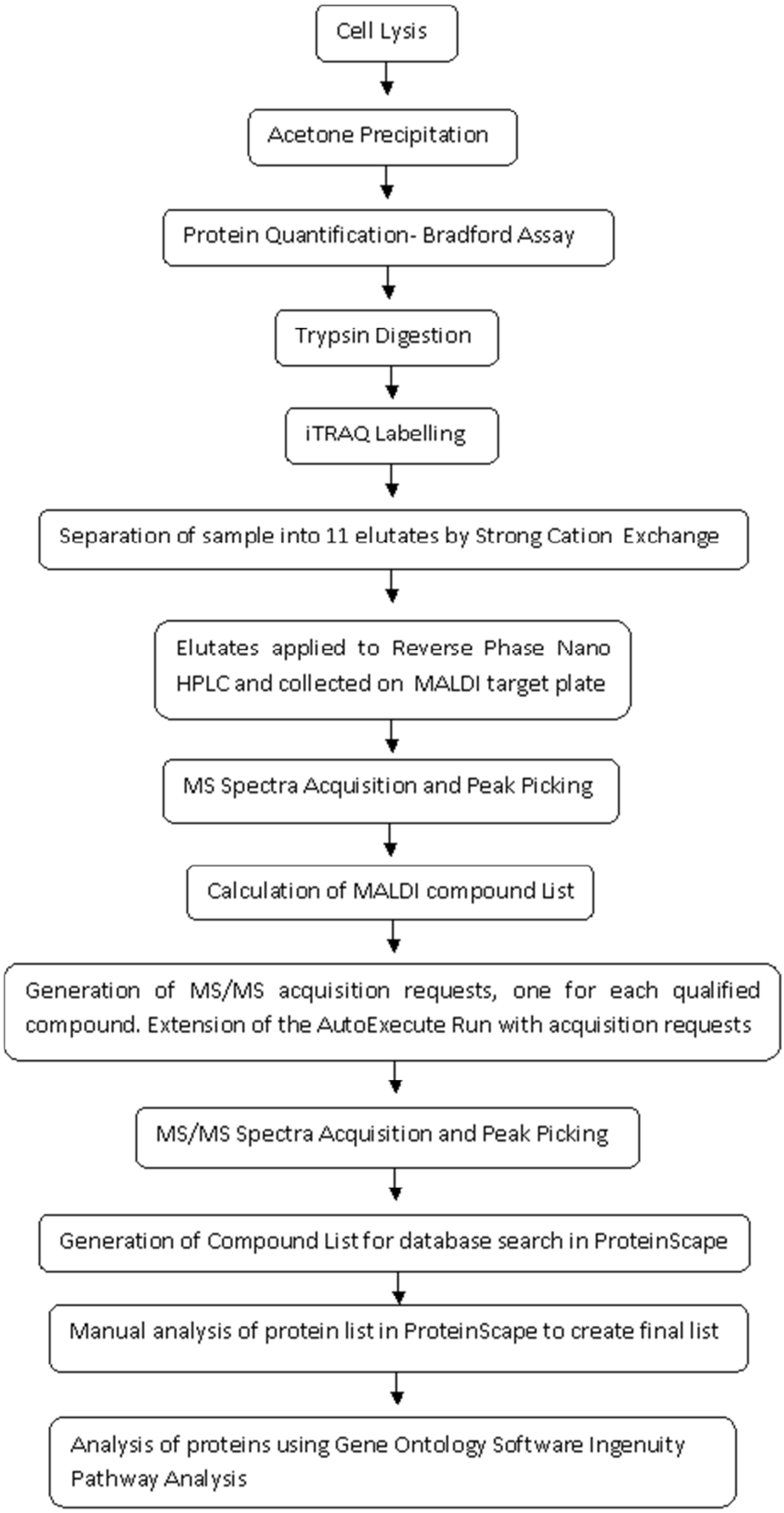


Figure 1. Workflow diagram to highlight the key steps in the proteomic experiment (modified from WARP-LC user manual, Version 1.2 2008)

2.2 Cell Lysis

Monolayers grown to 80% confluence were harvested via mechanical scrapping, washed 3 x with PBS before being pelleted by centrifugation (1000rpm, 5 minutes) and stored at -80^{o}C. Pelleted cells were thawed on ice and then re-suspended in 2x pellet volume of protein extraction buffer (7M urea, 2M thiourea, 4% CHAPS (3- ((3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid), 0.1% w/v SDS, 0.05% w/v sodium deoxycholate in PBS). Cells were then homogenised by sonication using 6 cycles, repeated 3 times and incubated on ice for 30 seconds between cycles. The sample was then centrifuged at 13,000rpm for 15 minutes at 4^{o}C after which the liquid phase was collected and stored at -20^{o}C. The Bradford assay was performed as described previously (Chapter 2, section 2.3.5) to measure the total protein concentration in each sample (see Appendix).

2.3 Acetone Precipitation

Once the protein concentration has been determined, 250µg of protein from each sample was added to 1ml of chilled acetone, briefly vortexed, centrifuged at 12,000rpm for 1 minute at room temperature, and incubated at -20^{o}C overnight. Following the overnight incubation samples were centrifuged again at 13,000rpm for 20 minutes at 4^{o}C before the supernatent was removed and pellets lyophilised at 40^{o}C. Once completed the acetone precipitated samples were re-suspended in 8M urea in 400mM ammonium bicarbonate and re-analysed with the Bradford assay to determine how much protein had been lost through acetone precipitation.

2.4 Protein Digestion

Dithiothreitol (1µl of 50mM) was added to the re-suspended samples (80µg of protein) before vortexing and centrifuging. The samples were incubated in a water bath at 60^{o}C for 15 minutes and then allowed to cool on ice for 5 minutes. Iodoacetamide (1µl of

100mM) was added before another brief vortex and centrifugation. The samples were then incubated in the dark at room temperature for 20 minutes before a final addition of 13µl of trypsin buffer (360mM ammonium bicarbonate, 10% acetonitrile) and 2µl trypsin (1mg/ml in trypsin buffer) Samples were left to incubate at 37^{o}C overnight.

Controls

Several controls were also included in the experiment. These consisted of the following:-

- Myoglobin and Trypsin- 18µl myoglobin, 2 µl trypsin, 2µl trypsin buffer.
- Trypsin alone-2 µl trypsin, 20µl trypsin buffer.
- Myoglobin alone-18µl myoglobin, 4µl trypsin buffer.

Trypsin was added at the same time to both the controls and the samples. After incubating at 37^{o}C for 20 hours an aliquot (0.5µl) of each reaction (sample and controls) was diluted 1 in 10 (in 10% acetonitrile), and analysed by manual MALDI MS (see section Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry).

2.5 Manual MALDI MS sample preparation

To determine whether trypsin digestion had been successful, samples were analysed manually on the Mass Spectrometer (MALDI-TOF/TOF UltraFlex II mass spectrometer, Bruker Daltonics, Bremen, Germany). The plate was first primed with matrix on each spot, followed by addition of 0.5µl of sample then 0.5 µl of matrix was added. Once the plate was dry it was analysed on the mass spectrometer.

2.6 Desalting the Samples

The samples were desalted on a C18 bond elution column (Kinesis Ltd, UK) using the following protocol. Methanol (1 ml) was first pushed through the column with a 10ml

syringe, followed by 2x1ml of Buffer A (2% acetonitrile, 0.05% trifluoroacetic acid) and then the sample. After this another 2x 1ml of Buffer A was used to remove non-bound buffer components. Finally the sample was eluted using 1x 1ml of Buffer B (80% acetonitrile, 0.05% trifluoroacetic acid). The samples were then vortexed and centrifuged (1000rpm, 1 minute) before lyophilization for 1hr at ~48^{o}C. Subsequently, the samples were re-suspended in 10µl of TEAB (triethylammonium bicarbonate with 0.1% SDS)

2.7 4-plex iTRAQ Labelling

The samples were labelled with iTRAQ labels for relative protein quantification. The labels are a set of 4 isobaric reagents which enables the multiplexing of up to 4 different samples within one LC/MS/MS run. The iTRAQ labels consists of 3 parts, a reporter group, a balance group and a peptide reactive group (Figure 2).

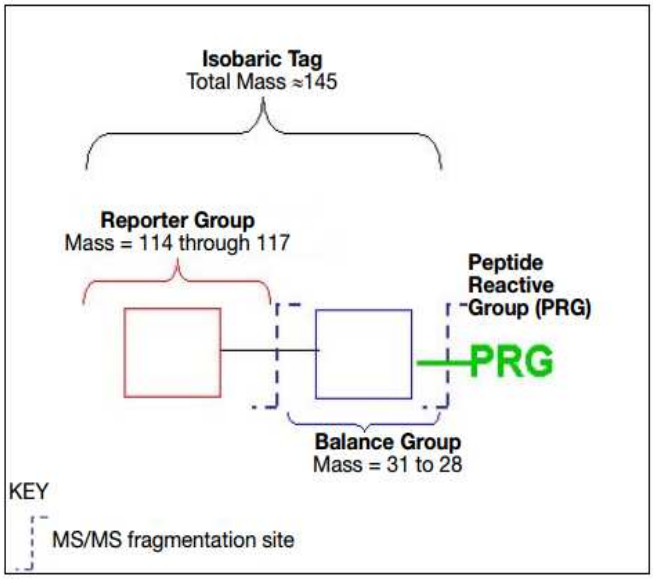


Figure 2. Structure of the iTRAQ reagents.[4]

Once the 4 iTRAQ labels (Applied Biosystems, ThermoFisherScientific, Leicestershire, UK) had defrosted, 40µl of ethanol was added to each tube and the contents of each added to the corresponding sample.

HT-29 (control) - iTRAQ 114

Necrotic Core Clone 1 - iTRAQ 115

Necrotic Core Clone 4 - iTRAQ 116

Necrotic Core Clone 5 - iTRAQ 117

A further 10µl of ethanol was added to each iTRAQ tube before vortexing, centrifugation and transfer to the appropriate samples. This was to ensure no reagent was lost. The pH was then tested to ensure it fell between pH7-10. The labelled samples were then incubated at room temperature for 2 hours before testing the pH again. If it had fallen outside the desired range it was adjusted with 1M TEAB. HPLC water (50µl) was then added to each labelled sample before combining all samples in one tube. Again to ensure no sample was lost, 25µl of HPLC water was added to each empty tube, vortexed, centrifuged (1000rpm, 1 minute) and then transferred to the combined samples. The combined sample was then lyophilised at ~50^{o}C (aqueous) and stored at -20^{o}C.

2.8 Strong Cation Exchange

Strong Cation Exchange (SCX) was used to separate the sample into fractions using the following protocol. The Isolute SCX column (Kinesis Ltd, UK) was washed by adding 1ml HPLC water, then 2x 1ml of cation exchange loading buffer (25% acetonitrile 10mM KH_2PO_4 in 25% acetonitrile, 0.01% sodium azide adjusted to pH 3.0 with HCl) was added and pushed through using a syringe. The sample was re-suspended in 600µl of cation exchange loading buffer and the pH measured and adjusted to pH 2.5-3.0 with 10% TFA (trifluoroacetic acid). Then the sample was added to the column and allowed to flow through using hydrostatic pressure.

The flow through was collected in an Eppendorf tube. The sample tube was washed with cation exchange buffer to minimise sample loss, the wash solution added to the column and flow through collected in the same Eppendorf tube. This first fraction was referred to as Flow Through 1 (FT1). Another 1ml of cation exchange loading buffer was added to the column and the flow through collected in a separate Eppendorf tube (FT2). To elute the peptides a range of elution buffers (cation exchange buffer + KCl) of differing potassium chloride concentrations were used to collect 11 separate fractions designated E01-11 as illustrated in Table 1 below:

Table 1. Potassium Chloride concentrations of the elution buffers and their corresponding elutions.

Eluate Fraction Number	Potassium Chloride Concentration (mM)
E01	30
E02	60
E03	90
E04	120
E05	150
E06	180
E07	240
E08	300
E09	500
E10	700
E11	1000

To elute the different fractions, 500µl of the corresponding elution buffer was added to the column, allowed to pass through via hydrostatic pressure and the eluate collected. To each collected fraction 1.5ml of C18 bond elution Buffer A was added to dilute the acetonitrile in the fractions before each fraction was again desalted on C18 cartridges

using the previously described method (section 2.6). All desalted fractions were then lyophilised and stored at -20^{o}C.

2.9 Reverse Phase Nano HPLC

Each fraction (E1-11) was re-suspended in 13µl of 10% acetonitrile and vortexed for 15 seconds, 12µl transferred to an LC vial and 5µl of this applied for LC-MALDI on an LC Packings Ultimate 3000 capillary HPLC system (Dionex, Camberley, Surrey, UK). The residual sample in the vial was then recovered and added to the 1µl of left over fraction, lyophilised and stored at -20^{o}C for future analyses. The sample was injected into the system using a 1µl sample loop and washed onto a C18 300µm x5mm, 5µm diameter, 100 Å Pep-Map column (LC Packings, Sunnyvale, CA, USA) using carrier solvent (0.05% TFA), before being transferred to a C18, 75m x15cm, 3µm 100 Å PepMap Column (LC Packings) equilibrated with 2% CH_3CN with 0.05% TFA (mobile phase A). Peptides were eluted over 105 minutes by modifying the mobile phase A to incorporate an increasing percentage (10-40%) of mobile phase B (80% acetonitrile, 0.05% TFA). The linear elution gradient produced 384 fractions of peptides (75nl each) which were collected, via a Proteineer FC Collector (Bruker Daltonics, Bremen, Germany), onto a MTP AnchorChip 800/384 target plate (Bruker Daltonics). Fractions were co-deposited with 1.2µl of a saturated solution of CHCA Matrix (α-cyano-4-hydroxy cinnamic acid, Sigma Aldrich, St. Louis, MO, USA). Matrix consists of 1.056ml 2:1 ethanol:acetone, 120µl CHCA saturated stock solution, 12µl 100mM ammonium phosphate and 12µl of 10%TFA. After the LC-MALDI run had finished 0.4µl of calibrant was hand-pipetted onto the plate between each group of four fractions before 1.5µl of matrix was added on top. The calibrant comprises of 5µl Peptide Calibration Standard II (Bruker Daltonics) diluted in 70µl 80% acetonitrile.

Table 2. List of all peptides comprising the Peptide Calibration Standard II and their molecular weights.

Peptide	Molecular Weight (Da)
Bradykinin fragment 1−7	757.86
Angiotensin II	1047.19
Angiotensin I	1297.49
Substrate P	1348.64
Bombesin	1620.86
Renin Substrate Tetradecapeptide porcine	1760.03
ACTH clip 1−17	2094.43
ACTH clip 18−39	2466.68
Somatostatin 28	3149.57

The plate was then analysed using MALDI mass spectrometry. Each SCX fraction was analysed twice on the HPLC creating two MTP Anchorchip target plates to be analysed on the mass spectrometer.

2.10 Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

MALDI MS analysis was carried out using a MALDI-TOF/TOF UltraFlex II mass spectrometer (Bruker Daltonics, Bremen, Germany) with a 200Hz smartbeam laser in reflectron positive ion mode. MS Data Acquisition was carried out using WarpLC software (v1.3) which was fully automated and included data acquisition (Flex Control v1.3) and data analysis (Flex Analysis v1.3 Snap peak detection algorithms). Initially MS analysis was performed which involved the accumulation of 500 shots in 100 shot steps within a 700-4200 Da mass range (minimum signal to noise of 7) to create a non redundant list of peptides for MS/MS analysis. External calibration was carried out during MS analysis once for every 4 spots on the plate. Once a non-redundant list of peptides had been created each individual peptide was subjected to MS/MS analysis

using LIFT mode (Flex Control v1.3) to acquire 1500 laser shots per spectrum. The fragment mass lists (Flex Analysis v1.3 using TopHat baseline subtraction and Savitzky-Golay smoothing) were compiled into a batch file before transfer to ProteinScape for database searching.

2.11 Protein Identification

Proteins were identified in ProteinScape v3.0 (Bruker Daltonics) using Mascot v2.4 (Matrix Science, UK) software, the MS/MS data was automatically compared against the 2013_02 SwissProt human protein database containing 20278 sequences (using the Bruker Biotools interface (v3.2 SR4). The Mascot search parameters included were trypsin digestion, two missed cleavage, no fixed modifications, fixed modifications of the carbamidomethylated cysteines, methionine oxidation and iTRAQ labelling on lysines and N-termini and possible iTRAQ side ractions. Peptide mass tolerance was set at 100ppm (parts per million) with a MS/MS tolerance of +/- 0.7 Da. A 95% confidence interval was included ($p<0.05$, Mascot score ≥ 28.0) for the purpose of searching the MS/MS data against SwissProt.

2.12 Data Analysis

ProteinScape was used to combine all 22 (11 fractions analysed in duplicate) LC-MALDI runs to create a single non-redundant protein list. The protein list was filtered manually to include only proteins identified by more than two first ranked unique peptides, at least one of which has a Mascot Score ≥ 28.0. All peptides not ranked first were removed from the list, all 1st ranked proteins regardless of Mascot score were included to provide additional iTRAQ values for quantitative analysis. In order to compare protein expression between the different groups, iTRAQ reporter ion ratios were determined for each protein NCC1/HT-29 = 115/114, NCC4/HT-29 = 116/114, NCC5/HT-29 = 117/114. For each group the mean ratio was determined and used to

normalise the data set by dividing each individual ratio by the mean to compensate for experimental variation in the preparation, trypsin digestion and iTRAQ labelling of the protein extracts. The standard deviations for each normalised group were then calculated and used to determine significance of the ratios (Table 3).

Table 3. Calculated Standard Deviations and subsequent Significance Ranges for iTRAQ reporter ion ratios for the three Necrotic Core Clones compared to the Parental Cell Line. The range of significant ratios includes values which are more than or equal to 1 standard deviation greater than 1, and equal to or less than the reciprocal of this first value. The lower range is derived from the reciprocal of the upper level of significance.

Ratio	Standard Deviation	Range of Significance
115/114	0.163	$1.163 \geq x \leq 1/1.163$ (0.860)
116/114	0.123	$1.123 \geq x \leq 1/1.123$ (0.890)
117/114	0.114	$1.114 \geq x \leq 1/1.114$ (0.898)

A ratio was deemed significant if they were greater or lesser than one standard deviation from the mean. The significant proteins were then transferred to Ingenuity Pathway Analysis (Ingenuity Systems, CA, USA) software for gene ontology analysis.

3 Results

3.1 Acetone Precipitation

Preliminary proteomic analysis using 8-plex iTRAQ yielded a low number of proteins (results not included) due limited quantities of sample and protein loss within the experimental procedure. The procedure was modified by (a) using 4-plex iTRAQ with fewer samples, (b) measuring the protein amount before and after acetone precipitation, (c) using a larger amount of starting material (250µg) to allow for protein loss during acetone precipitation

After the first Bradford assay 250µg of protein for each sample was acetone precipitated. Following this the Bradford Assay was repeated and a large percentage of the proteins were lost. The % yield ranged from 37.2% to 53.6% (Table 4).

Table 4. Amount of protein was measured in all four samples before and after acetone precipitation to determine the efficiency (yield) of the protein precipitation procedure.

Sample	Amount of protein pre acetone precipitation (µg)	Amount of protein post acetone precipitation (µg)	Yield (%)
HT29	250	103	41.2
NCC1	250	93	37.2
NCC4	250	116	53.6
NCC5	250	115	53.1

3.2 Trypsin Digestion

MS results from the 4 samples (HT-29, NCC1, NCC4, NCC5) show that trypsin digestion has taken place successfully and that the samples were ITRAQ labelled (Figure 3). In each sample, a complex mixture of signals was observed between m/z=

760 and 3522. Each was dominated by a strong signal at m/z= 1229.8 which was subsequently identified as the nondenaturing zwitterionic detergent CHAPS.

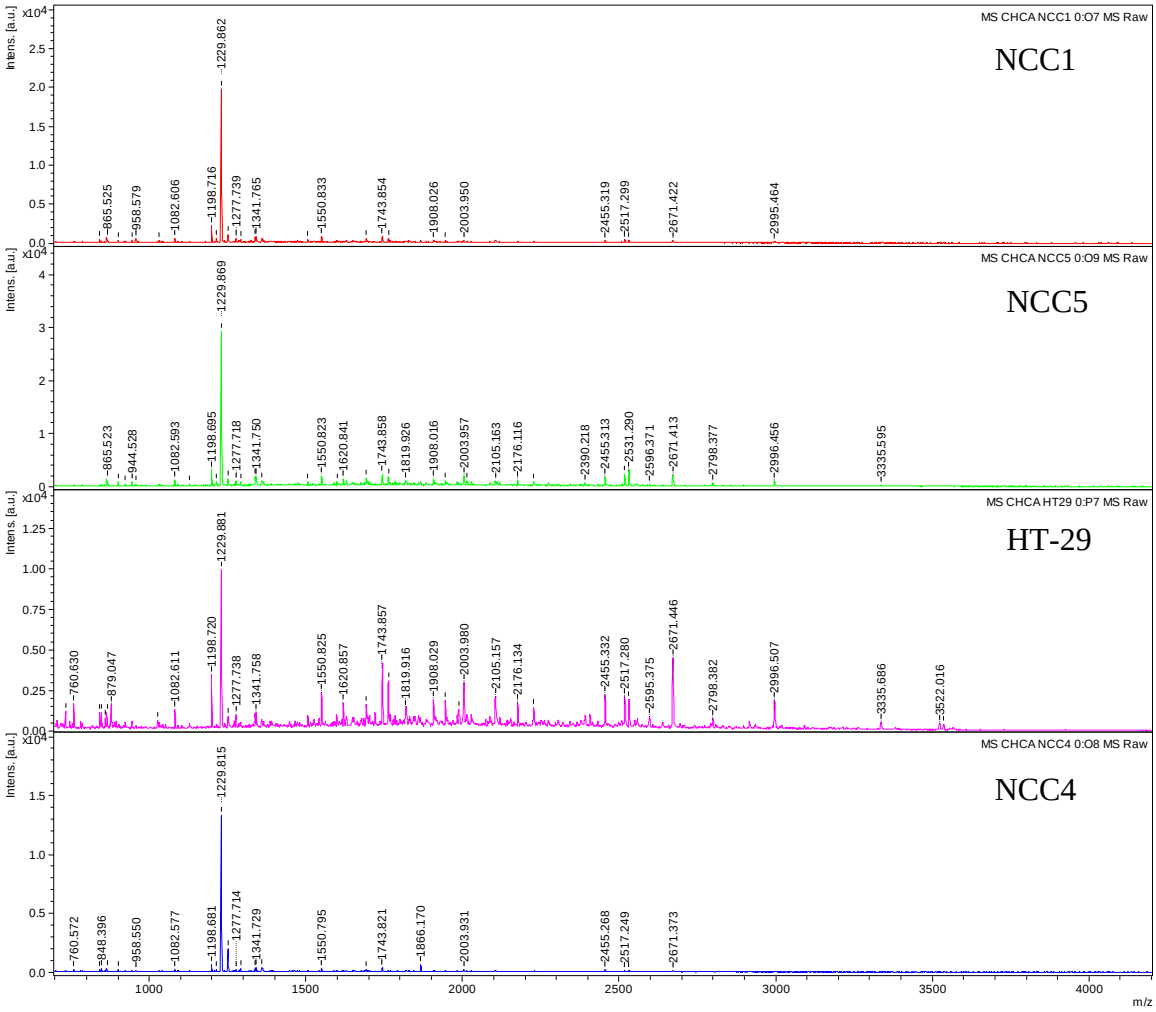


Figure 3. MS spectra of the individual trypsin digested protein extracts from HT29 parent cell line and clones NCC1, NCC4 and NCC5. The presence of multiple peaks demonstrates that effective trypsin digestion occurred in each of the 4 samples.

3.3 ITRAQ Labelled Proteins

Once the proteins from each of the four samples were labelled, the four samples were combined and the combined sample fractionated into 12 fractions (named elution (E)1-11, based on the order they were eluted from the column and FT1 collected from the flow through from the column) using strong cation exchange before each fraction underwent LC-MALDI. Each fraction was analysed twice and the results combined.

Table 5 shows the number of compounds found in each sample and the number of proteins these relate to for both LC-MALDI runs of each fraction.

Table 5. LC-MALDI results for each fraction separated by strong cation exchange. Each fraction was run twice and the results combined in Proteinscape.

	1st Run			2nd Run		
Fraction	Compounds for MS/MS	Background Compounds	Proteins	Compounds for MS/MS	Background Compounds	Proteins
E1	3019	430	786	4337	61	974
E2	4492	424	1178	6856	174	3295
E3	4724	207	1634	6471	318	1796
E4	5935	360	1998	6856	174	3295
E5	6258	331	1783	6977	78	3655
E6	5902	224	1746	8591	95	3885
E7	6066	30	1544	8034	87	3490
E8	6836	48	1804	7284	66	3219
E9	6767	40	1621	6669	167	1412
E10	6477	28	1330	7084	29	1390
E11	6419	11	1023	3652	0	597
FT1	2380	14	438	1694	59	320

The results of all LC MALDI data were combined to give a non-redundant list of 1432 proteins. Each protein was identified by at least 2 peptides one of which had a mascot score of >28.0. To identify any differences in expression of protein between the different samples, the ratios of the iTRAQ reporter ions were established for each protein.(HT29/NCC1, HT-29/NCC4 and HT-29/NCC5). Mean ratios were calculated for each of the three comparisons, and data was normalised using the calculated average. After normalisation of the results 134 proteins were found to be changed significantly in NCC1 compared to the parental cell line, with 61 found to be up-regulated and 73 down-regulated. In NCC4 222 proteins were found to be changed with

101 up-regulated and 121 down-regulated. And in NCC5 264 proteins were found at significantly different levels compared to the parental cell lines, of these 117 were found at higher levels and 147 were found at lower levels. Of these proteins 49 were found to be altered in all three of the clones (Figure 4) of which 21 were up-regulated and 28 were down-regulated. All proteins calculated to be significantly changed were analysed using Ingenuity Pathway Analysis software to look for commonly altered molecular pathways and networks.

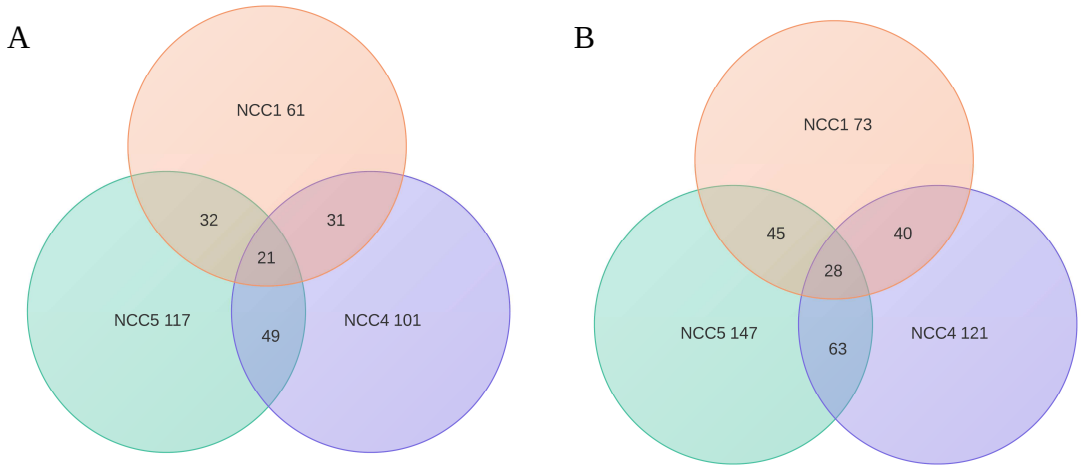


Figure 4. Venn Diagrams showing the overlap of the proteins found altered in all three necrotic core clones analysed in comparison to the parental cell line. Panel A details all the up-regulated proteins, panel B represents the down regulated proteins.

3.4 Necrotic Core Clone Analysis

The changed proteins in each of the individual clones was analysed in order to link any proteomic changes with the different functional capabilities the different clones possess. Following this the changed proteins common to all three clones were analysed in order to look for common mechanisms of cell survival, chemoresistance, metastasis and metabolism.

3.4.1 Necrotic Core Clone 1

As expected cancer was the top disease associated with the proteins found to be changed in NCC1 compared to the parental cell line with p-values ranging from 0.00000355 to 0.0157 for all different cancer types included. The top molecular functions found to be associated with the significant proteins included the following:-

1. Cellular Movement (p-value 0.00000835 - 0.0145) 29 proteins

2. Cell Death and Survival (p-value 0.00000933 - 0.0148) 46 proteins

3. Cell-To-Cell Signalling and Interaction (p-value 0.0000309E-05 - 0.0146) 14 proteins

4. Cellular Development (p-value 0.0000465E-05 - 0.0146) 30 proteins

5. Cellular Growth and Proliferation (p-value 0.0000659E-05 - 0.0137) 46 proteins

Using Ingenuity Pathway Analysis (IPA) nine networks were found to be significantly altered in Necrotic Core Clone 1 compared to HT-29 parental cells (Table 6).

ID	Molecules in Network	Score	Focus Molecul	Top Diseases and Functions
1	Akt, Alpha Actinin, Alpha catenin, Alpha tubulin, **↓ANXA2,** BCR (complex), **↓CD44,** **↓CHCHD2,** Collagen type I, **↑CRABP2,** **↑CTSB,** estrogen receptor, F Actin, **↑HSPB1,** IgG, **↑ISG15,** **↓KIF23,** **↑LAP3,** **↓MAP4,** **↓MARCKSL1,** NFkB (family), **↑PLAUR,** **↓POP7,** **↑PSMD13,** Rar, **↓S100A10,** **↑SERPINB5,** **↓SFN,** **↓SLC2A1,** **↑SNX5,** **↑SPINK1,** **↓STRN4,** trypsin, Ubiquitin, **↓ZYX**	43	22	Cell-To-Cell Signaling and Interaction, Connective Tissue Development and Function, Tissue Development
2	26s Proteasome, Actin, **↓ATPIF1,** **↓BAZ1A,** **↓BLVRB,** caspase, CD3, **↑CTSZ,** **↑DCTN4,** **↓DDX18,** **↓DDX19A,** **↑EIF2AK2,** **↑GM2A,** HISTONE, Histone H1, Histone h3, Histone h4, Hsp70, Hsp90, Ifn, IL12 (complex), Immunoglobulin, **↓KPNA3,** **↑MTA2,** NFkB (complex), **↑NUB1,** **↓PNP,** **↓POLR2B,** **↑PSAT1,** **↓PTMA,** Rb, RNA polymerase II, **↓SQSTM1,** **↑TGM2,** **↓TXN**	35	19	Cellular Compromise, Cell Death and Survival, Cancer
3	ABCB7, **↑ACO2,** ALG8, ALS2, **↑BCAS2,** **↓C9orf78,** COG4, **↑CPS1,** **↑CPSF2,** CSTF2T, **↓DHRS2,** EFTUD1, EXOSC5, FYTTD1, **↓HTATSF1,** MAU2, **↓MISP,** **↑NAE1,** **↓NDNL2,** NSMCE2, PRPS2, **↓PRPSAP1,** **↓RAB5B,** RABEPK, SGSM3, SIRT5, SMC6, **↑STX5,** STX17, **↑SUCLG1,** **↑SURF4,** TCEA2, TMED7, **↓TPD52L2,** UBC	29	16	Cellular Assembly and Organization, Cellular Function and Maintenance, Developmental Disorder
4	**↑ACAT1,** **↓ALB,** Alp, **↓ANXA1,** Ap1, calpain, **↑CES1,** Cpla2, Creb, **↓EPCAM,** ERK1/2, **↑FAF2,** **↑FARSA,** Fibrinogen, **↓GTF3C1,** HDL, IL1, Laminin, LDL, **↑LYZ,** Mek, **↓MUC5AC,** Mucin, Nos, **↓PDE12,** Pdgf (complex), PDGF BB, PLA2, **↑PLAA,** **↑PPT1,** **↓S100A11,** **↓SERPINA1,** **↑SNCG,** TCF, Tgf beta	28	16	Cancer, Gastrointestinal Disease, Hepatic System Disease
5	**↑AACS,** ADRB, AMPK, **↑CD3EAP,** **↑CRYZ,** Cyclin E, ERK, **↑FBP1,** FSH, **↑GLS,** **↑GSTP1,** Insulin, **↓KRT35,** Lh, **↓LSS,** Mapk, Nfat (family), **↑PDXK,** Pkc(s), PLC, Proinsulin, Rac, **↓RAP2B,** Ras, Ribosomal 40s subunit, Rnr, **↓RPS23,** **↓RPS24,** **↓RPS27,** Sos, **↓SPINT2,** TCR, **↑UBXN4,** **↑USF1,** **↓WFS1**	28	17	Drug Metabolism, Developmental Disorder, Gastrointestinal Disease
6	ACAD9, ACOT7, **↑ADI1,** AQR, ASNA1, BANF1, **↓C12orf45,** DAK, DCP2, **↓DDX27,** DDX42, FXN, **↑GLYR1,** MARK3, MST1R, **↓NHLRC2,** **↓NOL11,** **↓NUDCD1,** OSTF1, PLD3, **↓PMPCB,** **↑QSOX2,** **↓RAP2B,** **↑RBM22,** RUVBL2, SMG8, SMG9, TBC1D23, **↓TMPRSS13,** TYMP, UBC, USP36, **↓UTP18,** WDR1, **↓ZNF428**	24	14	RNA Damage and Repair, DNA Replication, Recombination, and Repair, Nucleic Acid Metabolism
7	beta-estradiol, BTAF1, Cathepsin, DLEU1, DNAJB2, **↑DNAJC2,** DNAJC4, **↓DUSP23,** **↑ECH1,** **↓EIF3J,** ESRRA, **↑GCHFR,** **↑GM2A,** GPN3, GPR35, **↓GPR37,** HTT, ID1, IL17RD, KAT5, **↓KIAA1324,** MIPEP, MYC, **↑NDUFA12,** **↑NUCB1,** PDX1, POLE2, **↓POLR2D,** PPP1R16B, RNA polymerase II, SLC9A8, TCF3, **↑TFAP4,** **↓TIMM50,** TNF	21	13	Cellular Assembly and Organization, Cell Cycle, Cancer
8	ABCB7, APP, **↓CNPY2,** **↑CRABP2,** **↓DNTTIP2,** **↑ECH1,** **↑GPT2,** HIST1H2BA, ICT1, IL5, KCNA4, KCNJ10, **↓MMAB,** MRPL11, MRPL20, MRPL24, **↑MRPS25,** MT-CYB, MTG1, NDUFS2, OLFM4, PDK3, PRDX5, **↑PSAT1,** **↓RBM8A,** RPS6KA6, RXRA, SCEL, Serpina3g (includes others), **↓SNTB1,** TPP1, UPF3A, **↑UQCRH,** UQCRQ, VDAC3	17	11	Free Radical Scavenging, Molecular Transport, Small Molecule Biochemistry
9	**↓AAMP,** ANKRD13B, **↓AP1S1,** Ap2 alpha, **↓C11orf58,** Calmodulin, Cg, Ck2, Collagen type IV, Collagen(s), DOK5, DUOX1, **↑EGFR,** Egfr-Erbb2, EGFR/PDGFR/IGFR, ER-α-Estradiol, Focal adhesion kinase, ganglioside GD2, Gsk3, Interferon alpha, Jnk, Mmp, P38 MAPK, PI3K (complex), Pka, **↑RNH1,** **↓RPLP1,** SH3PXD2B, **↑SLC25A13,** SUCNR1, Tnf (family), TNXB, TSH, Vegf, **↓YBX1**	11	8	Cell Death and Survival, Tumor Morphology, Dermatological Diseases and Conditions

Table 6. Networks found to be altered in Necrotic Core Clone 1 compared to the parental cell line. Number of molecules within the network found to be altered in the clone are shown, those in bold with either a green downwards arrow representing decreased expression, or a red upwards arrow representing increased expression. For each network a number of diseases and functions associated with the pathway are listed.

Of the nine networks found to be changed network 5 and 6 were found to interact (Figure 5). These were the pathways involved in drug metabolism and DNA repair and replication and involved the up regulation of various proteins known to be involved in chemoresistance such as sulfhydryl oxidase 2 (QSOX2), glutathione s-transferase (GST) and putative oxidoreductase (GLYR1).

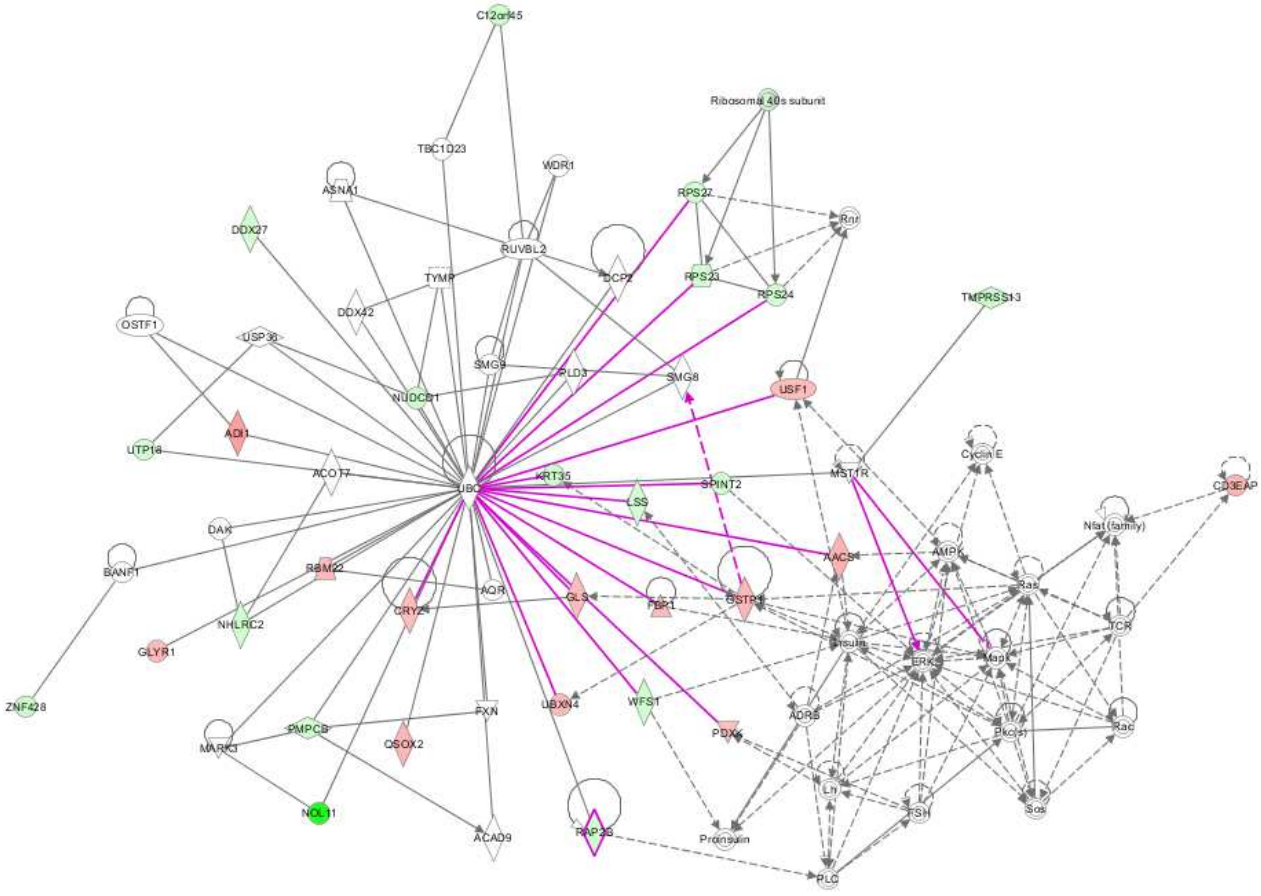


Figure 5. Diagram showing the interaction between Network 5 (Drug Metabolism, Developmental Disorder, Gastrointestinal Disease) and Network 6 (RNA Damage and Repair, DNA Replication, Recombination, and Repair, Nucleic Acid Recombination). Green shapes indicate proteins that are down-regulated, red shapes indicate proteins which are up-regulated. Pink lines indicate the point at which the two networks connect. For a key to what the different shapes represent refer to the appendix (Figure 4).

3.4.2 Necrotic Core Clone 4

As seen with NCC1, cancer was found to be one of the top diseases associated with the proteins found altered in NCC4 with a p-value between 0.000358 and 0.0219. Also similar to clone NCC1 the top molecular and cellular functions found to be altered included one of the same functions, cell death and survival. A summary of the cellular functions that may be altered as a result of differential protein expression are listed below.

1. RNA Post-Transcriptional Modification (p-value 0.000000000117 - 0.00866) 16 proteins

2. Cell Death and Survival (p-value 0.0000168 - 0.0219) 70 proteins

3. Free Radical Scavenging (p-value 0.0000502 - 0.0130) 18 proteins

4. Amino Acid Metabolism (p-value 0.000107 - 0.0219) 7 proteins

5. Cellular Growth and Proliferation (p-value 0.000107 - 0.0219) 63 proteins

Twelve networks were found to be altered in NCC4 (Table 7), within this group a theme was found. Several networks involved altered metabolism, including lipid metabolism and amino acid metabolism. Specific proteins involved in these networks, which were found at differing levels in NCC4 included SPINK1 also known as Tumour-associated Trypsin Inhibitor. This protein also found to be up-regulated in NCC1, is associated with increased invasion in multiple cancer types.

ID	Molecules in Network	Score	Focus Molecul	Top Diseases and Functions
1	**↓AGK, ↓ALB, ↓ALDH2, ↑ALDH1A1, ↑ATP6V1B1, ↓ATP6V1C1, ↑BANF1, ↓BECN1,** calpain, **↑CNN2, ↓CRAT, ↓CST3, ↓DSG2, ↑EIF5A,** ERK1/2, **↑GGH, ↓GTF3C1, ↓KRT9, ↑LAMTOR1, ↓LGALS3, ↓MUC5AC,** Mucin, **↓MVP,** NADPH oxidase, **↑PSAT1, ↑QTRTD1,** Rab5, **↑RAB11FIP1, ↓RAB5C, ↑SDCBP,** Secretase gamma, **↑SLC1A5, ↑SLC25A13,** TCF, **↓ZNF428**	54	28	Amino Acid Metabolism, Molecular Transport, Small Molecule Biochemistry
2	20s proteasome, 26s Proteasome, **↓AAMP, ↑AIP,** Akt, APC (complex), **↑CD3EAP, ↓CDC20, ↑CELF1, ↓CRIP2,** Cyclin E, **↑FAM129B,** G-Actin, **↓GM2A, ↓GSN,** Hsp70, Hsp90, **↓IGF2BP3, ↑PHF5A, ↑PHLDB1, ↓PPP1R8, ↑PPT1, ↑PSME2,** Rar, **↑S100A16, ↑SEC24B, ↓SEPT4, ↓SF3B1, ↑SNCG, ↑SRSF5, ↓TK1, ↑TMSB10/TMSB4X, ↑TRMT2A,** Ubiquitin, **↓ZYX**	46	25	RNA Post-Transcriptional Modification, Lipid Metabolism, Small Molecule Biochemistry
3	Actin, **↓AGR2, ↓ALDOA,** Alpha tubulin, **↓AP1S1,** Ap2 alpha, **↓BLVRB, ↑CFL1, ↓CKB, ↓CLINT1, ↑CRABP2, ↓DAK, ↑DNAJC2,** ERK, F Actin, **↓FTO, ↑GNL3,** Hdac, HSP, **↑HSPB1, ↑HSPH1,** IL12 (complex), Immunoglobulin, **↓LGALS4, ↓LMO7, ↑MATK,** Mek, **↓NHLRC2, ↓NSDHL, ↓POP7,** PP2A, **↑RPA3, ↓RTN4, ↓SOD1,** Sos	41	23	Cell Death and Survival, Cellular Function and Maintenance, Developmental Disorder
4	**↑ACTR2, ↑AHCYL1, ↓ALDOC,** Alp, Alpha catenin, **↓BID, ↑CALU,** CK1, **↑ESRRG, ↑GNL1, ↓GRN, ↓HEXIM1, ↓HTATSF1, ↓HTRA2,** Interferon alpha, ITPR, **↓LARP7, ↑LSM14B, ↓MAP4, ↑MT1E, ↑MTA2, ↓NDNL2,** NFkB (complex), P-TEFb, peptidase, **↑PLAA, ↓RTKN, ↑S100A4, ↑SNRNP200,** snRNP, **↓SNRPB2, ↑SNRPD1, ↑TRIM28, ↑UFM1, ↓WTAP**	40	26	RNA Post-Transcriptional Modification, Cellular Development, Nervous System Development and Function
5	**↓AACS,** ADRB, CD3, **↑CES1,** cytochrome C, **↓DDX18, ↓DDX19A, ↑EGFR,** estrogen receptor, **↑FABP5, ↑FTL,** GOT, Histone h3, IgG, Insulin, **↑KNDC1, ↓KRT20,** LDL, **↓LSS,** P38 MAPK, PI3K (complex), **↓POLR2B,** Proinsulin, **↑PSMD13, ↓PSMD14, ↑PYCARD,** Ras, Ras homolog, Sfk, **↑SLC25A22, ↓SQSTM1,** SRC (family), **↓STRN4,** TCR, **↓WFS1**	30	18	Organ Morphology, Skeletal and Muscular System Development and Function, Drug Metabolism
6	ALDOB, ATP5J, **↓CKB,** CRELD2, GEMIN6, GEMIN7, **↓GEMIN8,** GNG12, GUSB, **↓KIAA1324, ↓LACTB2,** MARK3, MDH1, **↓MRPL47, ↓MRPS18A, ↑MRPS18C, ↑MT1E,** NAGK, NDUFB9, **↓NOL11, ↓NUDCD1,** PDX1, PLD3, PPA1, **↑PPA2,** PTP4A1, **↑QSOX2, ↑SRSF8,** TYMP, UBC, USP36, **↑WDR66, ↓WDR75, ↓YIPF5, ↓ZNHIT2**	27	17	DNA Replication, Recombination, and Repair, Nucleic Acid Metabolism, Small Molecule Biochemistry
7	AASS, **↑CETN2, ↑CPS1,** CSF2, DIP2B, DNAJB2, **↑GGH, ↑GNL1, ↓GPR37,** GTPBP10, Histone h4, ICT1, KLHL5, **↓MMAB, ↑MRPL40, ↓MRPL47,** MRPS10, **↑MRPS25, ↓MRPS18A,** MST1R, MYC, **↑NCAPD2, ↓NOL11, ↑NUCB1,** PACRG, **↑PHF5A,** POC5, RAD21, SARS2, **↓SNTB1, ↓TMPRSS13,** UBC, UST, **↓XIRP2,** YARS2	27	17	Cancer, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function
8	**↑AASDHPPT,** Alpha Actinin, **↓ANTXR2,** Ap1, **↑BCAM,** Collagen type I, Collagen type IV, Collagen(s), Creb, **↑CTSB, ↓DCBLD2, ↑EHD1,** Focal adhesion kinase, Ifn, IL1, Jnk, Laminin, **↓MARCKSL1,** Mmp, **↑MYOZ1, ↓PDE12,** PDGF BB, Pka, PLC, PLC gamma, **↓S100A10, ↑SERPINB5, ↑SPINK1, ↓SYNE1,** Tgf beta, **↑TGM2, ↓TIMM8A,** Tnf (family), TSH, **↓TXN**	25	16	Organismal Functions, Tissue Development, Cardiovascular Disease
9	ATL2, **↓COPE,** CPNE8, **↓DHRS2,** ERGIC2, EXOSC5, HOXC13, **↓KRT35,** MZT1, **↓MZT2B, ↓NCBP2,** NCLN, **↓NDUFA12,** NDUFV3, NEDD1, **↑NOMO1 (includes others), ↑NUB1, ↓PDRG1,** PRICKLE3, RAB24, **↓RBM15, ↓RBM25, ↑RP2,** SACM1L, **↓SGPL1,** SIPA1L3, **↑SRSF6, ↑SRSF11,** TCEA2, THAP11, TUBG2, TUBGCP5, TUBGCP6, **↑UBAP2L,** UBC	25	16	Cellular Assembly and Organization, Cellular Function and Maintenance, RNA Post-Transcriptional Modification
10	ACAD9, **↑ADI1, ↑ARSE, ↓CCBL2, ↓CNPY2, ↑COMMD3,** COMMD4, COMMD6, COMMD8, COQ6, **↓FAM3C,** FXN, **↓GALE, ↓GLOD4, ↑GLYR1,** HECW1, **↓LARP4B,** MARK3, MYLIP, OSTF1, **↓PMPCB,** PNRC2, RABIF, SEL1L, SUMF1, TMEM259, TNK2, TRMT6, **↑TRMT10C, ↑TRMT61A,** UBC, **↑UBXN4,** USP36, **↓UTP18,** VTI1B	23	15	Developmental Disorder, Hereditary Disorder, Metabolic Disease
11	60S ribosomal subunit, **↓ACAA2,** APP, Beta Arrestin, beta-estradiol, **↓C12orf45,** C15orf39, **↓CST3,** CUL3, **↓DYNC1I2,** DYNLRB2, EEF1B2, FN1, **↓GALE, ↑GCHFR,** GNRH2, **↑GPT2, ↓HIST1H2BA,** IFNG, KIAA0101, **↑LARS, ↑NTPCR,** RPF2, RPL37, RPL13A, RPL26L1, **↓RPL37A, ↓RPLP1, ↑RPLP2,** RUVBL2, Serpina3g (includes others), **↓TIMM50,** TXNDC5, **↑UQCRH,** VSIG4	22	15	Cell-To-Cell Signaling and Interaction, Cardiovascular Disease, Developmental Disorder
12	ARL6IP5, Calmodulin, CASC3, caspase, CELSR1, Cg, Ck2, CNGB1, **↓DLG4,** DOC2B, FSH, **↓IMMT,** Lh, LPHN2, Mapk, MN1, **↓PABPN1,** Pkc(s), PLA2, Plc delta, PPAP2A, PROK1, PTPN21, **↓RBM8A,** RNA polymerase II, SLC4A4, SLC6A9, SOX7, SRD5A2, SSTR4, **↑STX5,** Troponin t, Vegf, **↓XRCC1, ↓ZFR**	8	7	Cell Cycle, Cell-To-Cell Signaling and Interaction, Nervous System Development and Function

Table 7. Networks found to be altered in Necrotic Core Clone 4. Number of molecules within the network found to be altered in the clone are shown, those in bold with either a green downwards arrow representing decreased expression, or a red upwards arrow representing increased expression. For each network a number of diseases and functions associated with the pathway are listed.

3.4.3 Necrotic Core Clone 5

As with the previous two clones, cancer was seen as a top disease associated with the changed proteins in NCC5, with a p-value of 0.000263E-04 - 0.0264. The top molecular and cellular functions shown to be altered in NCC5 showed commonality with the other two clones in that it included Cell Death and Survival. Cellular movement was also found to be altered in this clone, something that could be related to increased migration seen in the NCC5 wound healing assay results. In particular proteins such as Basal- cell adhesion molecule (B-CAM) and Aldehyde Dehydrogenase 1A1 (ALDH1A1) were found to be up-regulated in not only NCC5 but also NCC4, whilst no significant difference in levels were seen in the least migratory clone NCC1.

RNA Post-Transcriptional Modification (p-value 0.00000569 - 0.00259) 15 proteins

Cellular Growth and Proliferation (p-value 0.00000598 - 0.0264) 91 proteins

Protein Synthesis (p-value 0.0000142 - 0.0144) 35 proteins

Cell Death and Survival (p-value 0.0000478 - 0.0264) 85 proteins

Cellular Movement (p-value 0.000110 - 0.0220) 36 proteins

ID	Molecules in Network	Score	Focus Molecul	Top Diseases and Functions
1	↑**ACTR2,** ↓**ADAM9,** aldo, ↓**ALDOA,** ↓**ALDOC,** Alpha catenin, Alpha tubulin, ↑**ANP32A,** ↑**CALU,** Dynein, ERK1/2, Lfa-1, ↓**MAP4,** ↑**MRPL39,** ↓**MVP,** ↑**NAE1,** ↑**PSMD13,** Rab5, ↓**RAB5B,** ↓**RIC8A,** Sec23, ↓**SEC23B,** ↑**SEC23IP,** ↑**SEC24B,** ↓**SLC25A22,** ↑**SNX5,** ↓**SNX9,** ↑**STMN1,** ↓**STRN4,** ↓**TPD52L2,** ↑**TUBA1B,** ↑**TUBB2A,** ↑**UFM1,** ↑**VBP1,** ↓**ZYX**	48	27	Drug Metabolism, Lipid Metabolism, Small Molecule Biochemistry
2	60S ribosomal subunit, ↓**AAMP,** ↑**ACACA,** ↓**BTF3,** Ck2, ↑**CPSF2,** ↓**CRIP2,** ↓**DUSP23,** ↓**GRN,** Histone h3, ↑**HNRNPH3,** Holo RNA polymerase II, Hsp70, ↑**NAP1L1,** ↓**NPM1,** Pkc(s), ↓**POLR2B,** ↓**POLR2D,** ↑**PTGES3,** ↓**RBM4,** ↓**RBM8A,** Ribosomal 40s subunit, RNA polymerase II, Rnr, ↓**RPL8,** ↓**RPL29,** ↓**RPL35,** ↓**RPL37A,** ↓**RPS6,** ↓**RPS28,** ↑**RPS4X,** ↓**SMC2,** ↓**TAF13,** TSH, ↓**YBX1**	44	25	Cancer, Hematological Disease, RNA Post-Transcriptional Modification
3	Akt, ↓**BUD31,** Calcineurin A, Cbp/p300, ↓**CDC20,** ↓**CKB,** ↑**CNBP,** ↑**CSE1L,** ↑**CTNNBL1,** Cyclin A, Cyclin E, E2f, ↓**EBNA1BP2,** ↑**GM2A,** ↑**HEXIM1,** Histone H1, ↓**HTATSF1,** Importin alpha, ↓**KPNA3,** ↓**LYZ,** ↓**MARCKSL1,** ↑**MCM2,** ↑**MCM5,** ↑**NCAPD2,** ↓**NCBP2,** P-TEFb, PI3K (family), ↓**PPP1R8,** ↑**PPT1,** Rar, Rb, ↓**SF3A2,** ↑**SNRPA,** ↑**TGM2,** ↑**UBE2C**	39	23	Lipid Metabolism, Small Molecule Biochemistry, RNA Post-Transcriptional Modification
4	↓**AACS,** ADRB, AMPK, ↑**C12orf10,** ↓**CHCHD3,** ↑**CRABP2,** cytochrome C, ↑**DNAJC2,** ↓**DNAJC11,** ↓**FABP5,** HSP, ↑**HSPA14,** ↑**HSPB1,** IFN Beta, IFN type 1, IgG, ↑**ISG15,** ↓**LAD1,** MHC Class II (complex), ↓**MTX2,** ↑**NACA,** PARP, ↑**PGD,** PI3K (complex), ↓**POP7,** ↓**SFN,** ↑**SNCG,** ↓**SRM,** ↑**TARS,** Tlr, ↓**TPP2,** ↓**TRIM25,** ↓**UBE2D3,** Ubiquitin, ↓**XRCC1**	39	23	Dermatological Diseases and Conditions, Drug Metabolism, Endocrine System Development and Function
5	↓**ALB,** ↑**ALDH1A1,** ↓**ANTXR2,** ↑**ATP2A2,** ↓**ATPIF1,** ↓**BAZ1A,** calpain, Creb, ↓**CST3,** Ctbp, ↓**DSG2,** Fcer1, ↓**GCLC,** Hdac, HISTONE, ↑**KDM1A,** ↓**KRT9,** ↓**LGALS3,** MAP2K1/2, ↑**MT1E,** ↑**MTA2,** ↑**MTDH,** ↓**MUC5AC,** Mucin, NFkB (complex), NuRD, ↑**PLAA,** Pro-inflammatory Cytokine, ↑**RBBP4,** ↑**RTKN,** Secretase gamma, TCF, ↑**USF1,** ↓**WTAP,** ↓**ZGPAT**	35	22	Cell Death and Survival, Cell Cycle, Cellular Assembly and Organization
6	Alp, ↑**BCAM,** ↓**BECN1,** Collagen Alpha1, Collagen type I, Collagen type III, Collagen type IV, Collagen(s), ↑**CTSB,** ↓**DCBLD2,** Focal adhesion kinase, Growth hormone, ↑**GSS,** ↓**HMGB1,** Integrin, ↓**ITGA2,** Jnk, Laminin, Pdgf (complex), PDGF BB, phosphatase, ↑**PLAUR,** ↑**PSAT1,** Rac, ↓**RHOG,** ↑**RNH1,** ↓**RRBP1,** ↓**S100A10,** ↑**SERPINB5,** ↑**SLC25A13,** Sos, ↓**SPINK1,** ↑**STXBP2,** Tgf beta, ↑**VAPB**	27	18	Cancer, Cellular Movement, Tumor Morphology
7	ACAD9, ↑**ADI1,** CETN2, ↑**COMMD3,** COMMD4, COMMD6, COMMD8, DCTPP1, ↓**DDI2,** ↓**DDX27,** DNTTIP2, FAM129B, ↑**GLYR1,** ↓**HIST1H2BA,** ↑**ITPA,** ↓**LARP4B,** ↓**LPCAT1,** ↓**LPCAT2,** ↑**MCCC2,** ↑**MPST,** MRPL23, ↑**MRPL40,** MRPS21, NAA15, OSTF1, ↓**PMPCB,** SARS2, TBL2, ↑**TMEM43,** TOR1A, ↑**TOR1AIP1,** TOR3A, ↑**TRMT10C,** UBC, USP44	26	17	Developmental Disorder, Hereditary Disorder, Metabolic Disease
8	Actin, ↓**AGR2,** ↓**AHCYL1,** Alpha Actinin, CD3, ↑**CFL1,** Cofilin, Cyclin B, ↑**DCTN4,** ↓**DDX19A,** ERK, F Actin, JINK1/2, ↑**LAMTOR1,** Ldh, ↓**LGALS4,** Limk, ↑**MATK,** Mek, Mlc, ↑**MYL12B,** ↑**MYOZ1,** PP1 protein complex group, PP2A, ↑**PRKCD,** Ras homolog, ↑**ROCK2,** Rock, Rsk, ↓**S100A11,** ↓**SPINT2,** ↓**TES,** ↑**TMSB10/TMSB4X,** ↑**TXNDC17,** ↑**UBA6**	25	18	Cellular Assembly and Organization, Developmental Disorder, Gastrointestinal Disease
9	ACIN1, ATF7, ATP5J, BANF1, ↓**C21orf59,** ↓**CCDC58,** CDK5RAP3, CHAF1B, DDX56, ↓**GGCT,** GNB1, ↓**HEATR5B,** HIST1H1D, KRT16, ↓**LACTB2,** MARK3, ↑**MRPS25,** ↓**MRPS18A,** ↑**MRPS18C,** ↑**MTA2,** NAGK, ↓**NOL11,** NUMA1, ↑**OCIAD1,** ↓**PSTK,** ↑**QSOX2,** RECQL, SUB1, TRA2B, TUFM, UBC, ↓**WDR75,** XPC, ↓**ZNF428,** ↓**ZNHIT2**	24	16	Cellular Assembly and Organization, Dermatological Diseases and Conditions, Hereditary Disorder
10	↑**AASDHPPT,** ↓**ACAA1,** ↓**ACAA2,** acetyl-CoA C-acyltransferase, ↓**ANLN,** AP5Z1, ↓**C9orf78,** ↑**DUT,** EFTUD1, ↓**EIF3J,** ↓**ESD,** ↓**FKBP10,** HERC4, HOXC13, ↓**IRF2BP1,** ↓**KRT35,** MZT1, ↓**MZT2B,** NEDD1, ↓**NOC4L,** NUBP2, PEX7, PRICKLE3, RAB24, SEC63, ↑**SRSF11,** ↑**SURF4,** THAP11, TMED7, ↑**TRMT2A,** TUBG2, TUBGCP5, TUBGCP6, TXNDC11, UBC	24	16	Cellular Assembly and Organization, Cellular Function and Maintenance, Cell Cycle
11	↑**API5,** ↓**ATP5D,** ↓**C11orf58,** ↓**C12orf45,** C4orf27, ↓**DAK,** DIP2B, DNAJB2, FOCAD, ↓**GPR37,** ↓**MISP,** ↑**NHLRC2,** ↑**NTPCR,** PACRG, ↓**PDE12,** ↑**PPP4R1,** RAD21, RNF213, RUVBL2, SCRN1, ↓**SMC3,** SMC1-SA2, SMG8, SMG9, SSU72, STAG1, STAG3, TBC1D23, ↓**TFAP4,** THYN1, ↑**TRA2A,** UBC, WFDC5, ↓**XIRP2,** ↓**ZNHIT2**	24	16	Developmental Disorder, Hereditary Disorder, Neurological Disease

Table 8. Networks found to be altered in Necrotic Core Clone 5. Number of molecules within the network found to be altered in the clone are shown, those in bold with either a green downwards arrow representing decreased expression, or a red upwards arrow representing increased expression. For each network a number of diseases and functions associated with the pathway are listed.

11	↑**API5,** ↓**ATP5D,** ↓**C11orf58,** ↓**C12orf45,** C4orf27, ↓**DAK,** DIP2B, DNAJB2, FOCAD, ↓**GPR37,** ↓**MISP,** ↑**NHLRC2,** ↑**NTPCR,** PACRG, ↓**PDE12,** ↑**PPP4R1,** RAD21, RNF213, RUVBL2, SCRN1, ↓**SMC3,** SMC1-SA2, SMG8, SMG9, SSU72, STAG1, STAG3, TBC1D23, ↓**TFAP4,** THYN1, ↑**TRA2A,** UBC, WFDC5, ↓**XIRP2,** ↓**ZNHIT2**	24	16	Developmental Disorder, Hereditary Disorder, Neurological Disease
12	26s Proteasome, Ap1, ↑**BAIAP2L1,** BCR (complex), ↓**BLVRB,** ↑**CALB2,** caspase, ↑**EIF5A,** estrogen receptor, ↑**GLS,** ↓**HADH,** Histone h4, ↑**HLA-A,** ↑**HLA-C,** Hsp90, Ifn, Ifn gamma, Igm, IL1, IL12 (complex), Immunoglobulin, Interferon alpha, ↓**IPO5,** Mapk, MHC Class I (complex), MHC CLASS I (family), ↑**NUB1,** P38 MAPK, ↓**PSMF1,** ↓**PTMA,** ↓**SQSTM1,** SRC (family), ↑**STAT1,** TCR, Tnf (family)	20	14	Amino Acid Metabolism, Post-Translational Modification, Small Molecule Biochemistry
13	↓**ADAM9,** APP, AQP5, ↑**ARSE,** beta-estradiol, CDCA7, CDK18, COQ6, ↑**CRABP2,** CSF2, ↓**CST3,** ↓**DHRS2,** DMTN, EGFR, FGF7, GNRH2, ↑**GPT2,** KCNAB1, ↓**LAD1,** Ldb3, ↓**LPCAT1,** ↓**LPCAT2,** MAPK1, ↓**NDUFB10,** OSBPL3, ↓**SCAMP3,** Serpina3g (includes others), SNCA, ↓**SNTB1,** SPRED2, TMEM87A, TNK2, TPD52L1, ↑**UROD,** ↓**UTP18**	19	14	Drug Metabolism, Endocrine System Development and Function, Lipid Metabolism
14	ADCY8, ARL6IP5, ↓**ATPIF1,** Calmodulin, ↓**CES1,** CLIP2, DUOX1, ↓**EIF5B,** Enolase, FSH, GNRH2, GPR56, Gsk3, HSD3B1, HSD3B2, ↓**IDH3B,** Insulin, ↑**LARS,** LDL, Lh, MAP6, PDXK, Pka, PPP1R3D, Ral, Ras, ↑**SPR,** ↓**TIMM50,** ↓**TIMM8A,** Tni, TUBA3E, Tubulin, Vegf, ↓**WFS1,** ↓**YWHAZ**	12	10	Drug Metabolism, Endocrine System Development and Function, Lipid Metabolism
15	KIAA0196, ↓**KIAA1324,** LSM1, LSM2, LSM3, ↑**LSM14B,** ↓**LTA4H,** MRPL11, ↓**NHP2L1,** NOP10, PDX1, PPIH, PRPF3, PRPF6, PRPF31, RNU4-1, RRP9, SART1, ↓**SDHA,** SDHC, snRNP, SNRPA1, ↑**SNRPA,** SNRPB2, SNRPC, SNRPN, TGS1, THUMPD1, UBC, URB1, WDR43, WRAP53, ZNHIT3, ZNHIT6, ZP3	6	6	RNA Post-Transcriptional Modification, Dermatological Diseases and Conditions, Developmental Disorder

Table 8 Continued.

3.5 Common Analysis

There were many similarities between the analyses of the three clones. All three had alterations in cellular growth and proliferation, which correlated with growth characteristics observed when clones were grown in culture (Chapter 1, Figure 20). A large number of proteins found altered in all of the clones were found to be related to cell death and survival (Table 9). In total 49 proteins were found to be changed in all three clones (21 up-regulated and 28 down regulated see Figure 4) and these proteins were analysed using ingenuity pathways analysis software to determine the top cellular and molecular functions associated with the proteins:

Cell Death and Survival (p-value 0.0000376 - 0.0471) 22 proteins

Cellular Compromise (p-value 0.000440 - 0.0387) 8 proteins

Cellular Assembly and Organization (p-value 0.000872 - 0.0492) 11 proteins

Cellular Movement (p-value 0.000995 - 0.0456) 11 proteins

Lipid Metabolism (p-value 0.00109 - 0.0492) 9 proteins

The top function that all three clones had in common was cell death and survival (involving 22 out of the 49 proteins). There were 4 networks found to be altered in all 3 clones, the individual proteins involved and their exact effort (positive or negative) on the described function while be expanded upon subsequently.

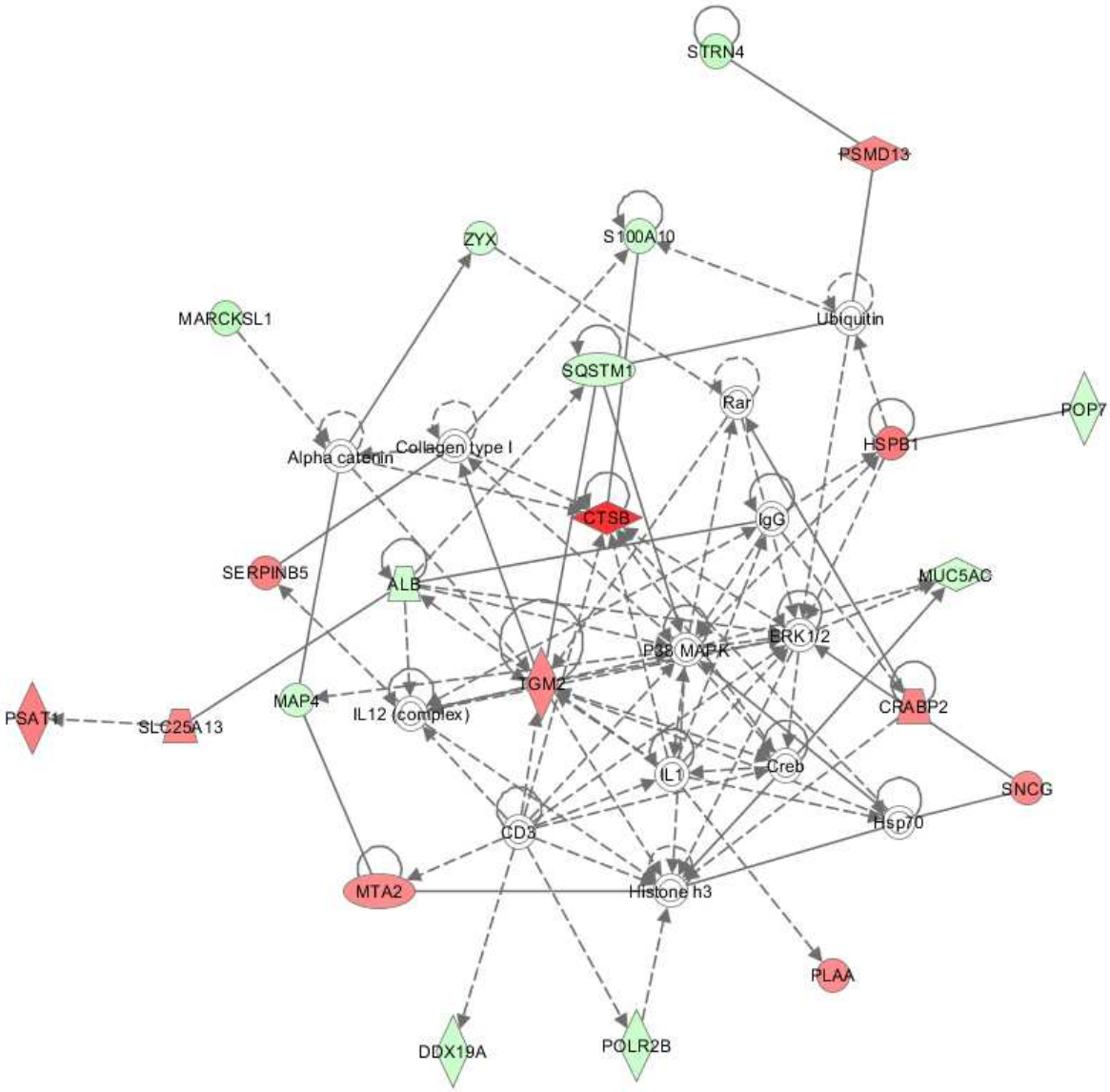


Figure 6. Top network identified by IPA analysis. Network 1: Cell Death and Survival, Renal Damage, Renal Tubule Injury. Solid arrows represent known interactions, dotted arrows represent indirect interactions. Red shapes are proteins seen at increased levels, green shapes are proteins found at decreased levels compared to the parental cell line. For a key to what the different shapes represent refer to the appendix (Figure 4).

Figure 6 shown above presents the cell death and survival network which was found to be altered in all three of the clones. In order to understand whether the network was altered in a beneficial way which may help explain the survival of the clones within the necrotic core the individual proteins within the network were investigated for relevance. TGM2 (Tissue Transglutaminase 2) was found to be increased in the clones, this protein has previously been shown to be elevated in CD44 cells and the inhibition of which

increased cell death in glioma patient derived tissues.[5] Also involved in the cell death and survival pathway was Cathepsin B (CTSB) a thiol protease, up-regulated in all three clones. Cathepsin B has been linked to poor prognosis and increased metastatic and invasive phenotype in human tumours. Metastasis associated protein 2 (MTA2) was also found at increased levels in all three clones. Increased expression of this protein is associated with invasion and metastasis in gastric cancer.[6] Sequestosome-1 (SQSTM1) also known as p62 is involved in autophagy, but was found to be down regulated in all three clones.

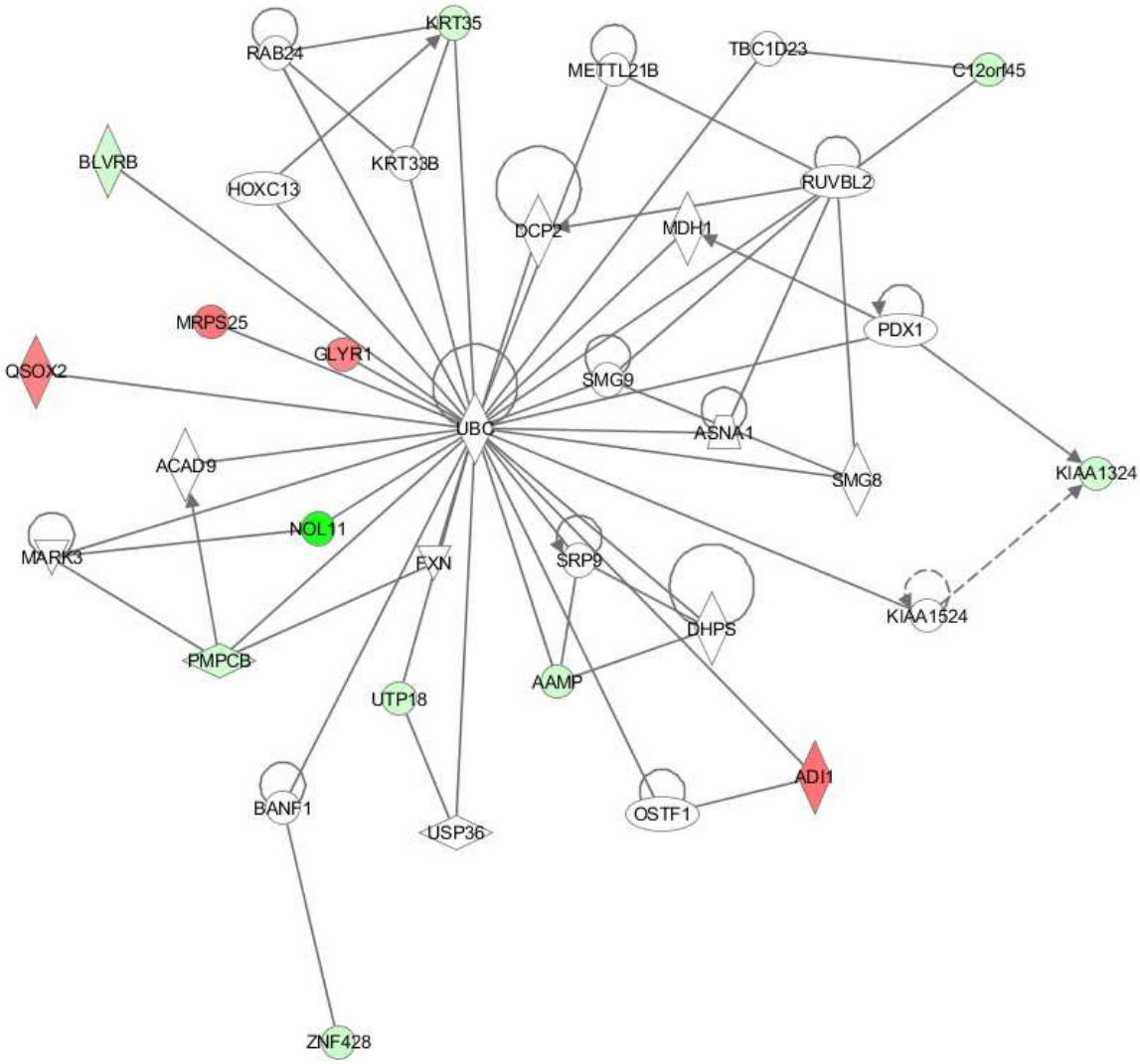


Figure 7. Network 2: RNA Damage and Repair, Hereditary Disorder, Cell Cycle. Solid arrows represent known interactions, dotted arrows represent indirect interactions. Red shapes are proteins seen at increased levels, green shapes are proteins found at decreased levels compared to the parental cell line. For a key to what the different shapes represent refer to the appendix (Figure 4).

The Cell cycle pathway was also found to be altered (Figure 7), with a total of 13 proteins found to be up- or down-regulated. Quiescin sulfhydryl oxidase 2 (QSOX2) an enzyme involved in the oxidation of sulfhydryl groups, was seen at higher levels in all three clones compared to the parental cell line. Glyoxylate reductase 1 (GLYR1) was also found to be up regulated in this pathway and is known to be involved in regulating p38 MAP kinase a key protein involved in the regulation of the cell cycle in response to stress.[7]

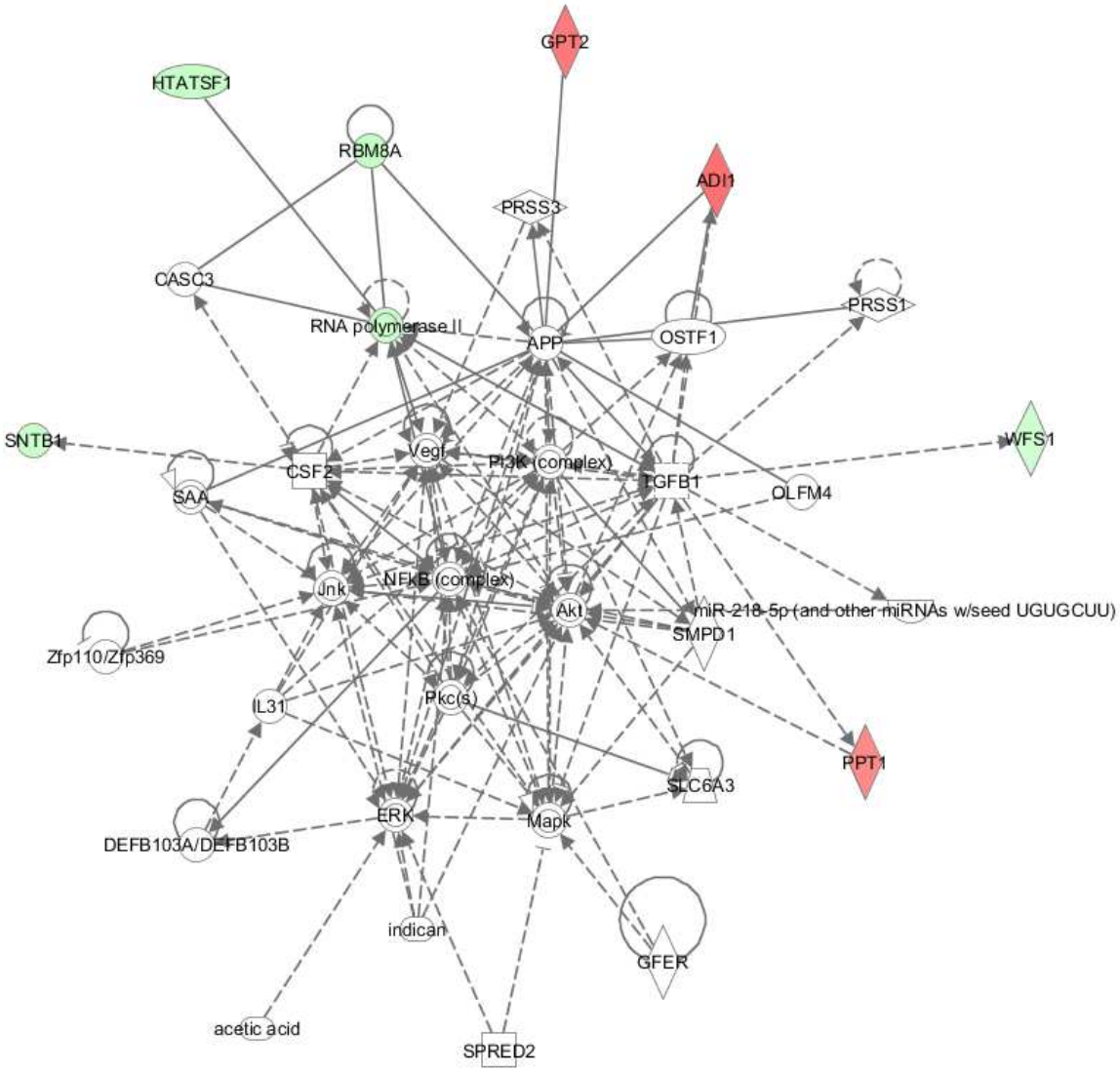


Figure 8. Network 3: Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry. Solid arrows represent known interactions, dotted arrows represent indirect interactions. Red shapes are proteins seen at increased levels, green shapes are proteins found at decreased levels compared to the parental cell line. For a key to what the different shapes represent refer to the appendix (Figure 4).

The network involved in lipid metabolism and small molecule biochemistry was also found to be altered in all three clones (Figure 8). Proteins involved in a variety of different metabolism related processes were shown to be altered. These included up-regulation of Palmitoyl-protein thioesterase 1 (PPT1) involved in lysosomal degradation and Alanine aminotransferease (ALAT2) also known as glutamic pyruvate transaminase a protein involved in glutaminolysis.[8]

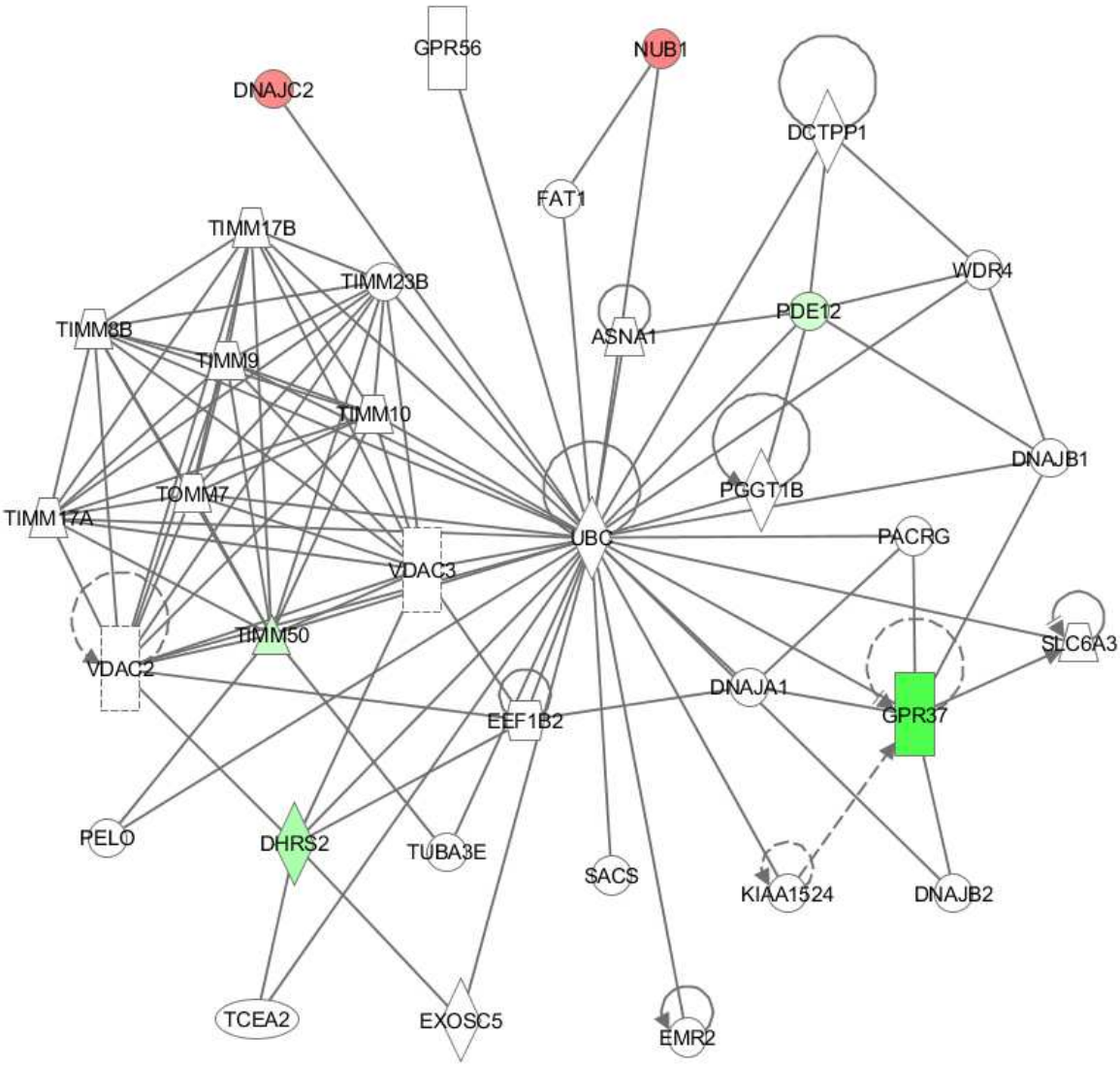


Figure 9. Network 4: Cellular Assembly and Organization, Cellular Compromise, Drug Metabolism . Solid arrows represent known interactions, dotted arrows represent indirect interactions. Red shapes are proteins seen at increased levels, green shapes are proteins found at decreased levels compared to the parental cell line. For a key to what the different shapes represent refer to the appendix (Figure 4).

In the Cellular Assembly and Organisation, Cellular Compromise and Drug Metabolism pathway shown in Figure 9 a number of proteins were also found to be altered. DNAJC15 also known as MCJ (methylation-controlled J protein) resides on the inner mitochondrial membrane and is involved in negatively regulating mitochondrial membrane potential and the production of ATP. [9]

Table 9 List of all up- or down-regulated proteins found common to all 3 clones. Green ratios represent the cases where the protein was found at increased levels in the clones compared to the parental cell line, and red represents the cases where the clones' expression was decreased compared to the parental cell line.

Accession	Protein	MW	Score	# Peptides	Ratio NC1/HT-29	Ratio NC4/HT-29	Ratio NC5/HT-29
Cell Death and Survival							
STRN4	Striatin-4	80.5	88	2	0.400	0.830	0.633
MRP	MARCKS-related protein	19.5	87	1	0.416	0.820	0.568
RPB2	DNA-directed RNA polymerase II subunit RPB2	133.8	234	4	0.714	0.773	0.680
S10AA	Protein S100-A10	11.2	109	2	0.745	0.868	0.829
DD19A	ATP-dependent RNA helicase DDX19A	53.9	81	2	0.761	0.859	0.587
MAP4	Microtubule-associated protein 4	120.9	153	5	0.831	0.820	0.829
SQSTM	Sequestosome-1	47.7	336	6	0.831	0.878	0.838
ZYX	Zyxin	61.2	305	7	0.831	0.878	0.875
POP7	Ribonuclease P protein subunit p20	15.6	66	1	0.847	0.859	0.773
MUC5A	Mucin-5AC (Fragments)	526.3	150	5	0.847	0.754	0.838
ALBU	Serum albumin	69.3	102	3	0.855	0.735	0.810
SERC	Phosphoserine aminotransferase	40.4	147	3	1.458	1.202	1.210
HSPB1	Heat shock protein beta-1	22.8	361	6	1.396	1.269	1.322
CATB	Cathepsin B	37.8	45	2	1.396	2.719	2.104
RABP2	Cellular retinoic acid-binding protein 2	15.7	588	11	1.309	1.212	1.155
SPB5	Serpin B5 OS=Homo sapiens GN=SERPINB5 PE=1 SV=2	42.1	113	3	1.255	1.164	1.304

PSD13	26S proteasome non-ATPase regulatory subunit 13	42.9	85	2	1.278	1.154	1.155
MTA2	Metastasis-associated protein MTA2	75	196	5	1.231	1.126	1.145
TGM2	Protein-glutamine gamma-glutamyltransferase 2	77.3	563	15	1.215	1.231	1.182
SNCG	Gamma-synuclein	13.3	283	4	1.207	1.231	1.117
CMC2	Calcium-binding mitochondrial carrier protein Aralar2	74.1	313	6	1.192	1.126	1.238
PLAP	Phospholipase A-2-activating protein	87.1	82	3	1.184	1.154	1.164
Cellular Assembly and Organisation, Cellular Compromise and Drug Metabolism							
GPR37	Probable G-protein coupled receptor 37	67.1	145	6	0.196	0.200	0.242
DHRS2	Dehydrogenase/reductase SDR family member 2	27.4	111	1	0.274	0.286	0.810
TIM50	Mitochondrial import inner membrane translocase subunit TIM50	39.6	119	2	0.721	0.782	0.633
PDE12	2',5'-phosphodiesterase 12	67.3	141	4	0.855	0.849	0.894
NUB1	NEDD8 ultimate buster 1	70.5	101	1	1.200	1.240	1.276
DNJC2	DnaJ homolog subfamily C member 2	72	231	4	1.184	1.126	1.201
Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry							
HTSF1	HIV Tat-specific factor 1	85.8	199	5	0.627	0.782	0.698
RBM8A	RNA-binding protein 8A	19.9	73	1	0.690	0.668	0.661
SNTB1	Beta-1-syntrophin	58	117	1	0.776	0.859	0.652
WFS1	Wolframin	100.2	98	1	0.831	0.811	0.838
ALAT2/	Alanine aminotransferase 2	57.9	216	6	1.356	1.355	1.294

GPT2 PPT1	Palmitoyl-protein thioesterase 1	34.2	175	3	1.168	1.154	1.229
RNA Damage and Repair, Cell Cycle							
NOL11	Nucleolar protein 11	81.1	82	1	0.165	0.191	0.149
CL045	Uncharacterized protein C12orf45	20.1	91	1	0.721	0.830	0.894
ZN428	Zinc finger protein 428	20.5	89	3	0.745	0.716	0.717
AAMP	Angio-associated migratory cell protein	46.7	72	1	0.761	0.868	0.782
BLVRB	Flavin reductase (NADPH)	22.1	126	7	0.761	0.868	0.885
KRT35	Keratin, type I cuticular Ha5	50.3	174	4	0.815	0.878	0.875
K1324	UPF0577 protein KIAA1324	111.3	113	2	0.823	0.859	0.819
UTP18	U3 small nucleolar RNA-associated protein 18 homolog	62	94	3	0.847	0.878	0.708
MPPB	Mitochondrial-processing peptidase subunit beta	54.3	200	6	0.847	0.868	0.857
MTND	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	21.5	67	1	1.694	1.269	1.313
RT25	28S ribosomal protein S25, mitochondrial	20.1	29	1	1.349	1.240	1.536
QSOX2	Sulfhydryl oxidase 2	77.5	153	4	1.247	1.231	1.248
GLYR1	Putative oxidoreductase GLYR1	60.5	44	1	1.207	1.145	1.285
RRMJ3	pre-rRNA processing protein FTSJ3	96.5	93	2	1.278	1.297	1.406
PAP1M	Polyadenylate-binding protein 1-like 2	22.8	186	3	1.184	1.154	1.266

Further analysis was also performed on the list of changed proteins to look at their downstream effects. Two similar processes were found to be activated as a result of the changes seen in the proteome of the necrotic core clones (Figure 10). Changes in migration was shown to be a downstream effect caused by increased expression of Cathepsin B, Heat shock protein beta-1, Protein-glutamine gamma-glutamyltransferase 2 and Synuclein gamma, and the decreased expression of inhibitory proteins MARCKS related protein and Zyxin. Similarly, metastasis was also calculated to be up-regulated as a downstream effect of the protein changes seen. This also involved the increased expression of Cathepsin B and Synuclein gamma with the addition of Tissue Transglutaminase 2. The combination of these results is consistent with the clones possessing a more migratory and metastatic phenotype as shown by the wound healing assay results presented previously.

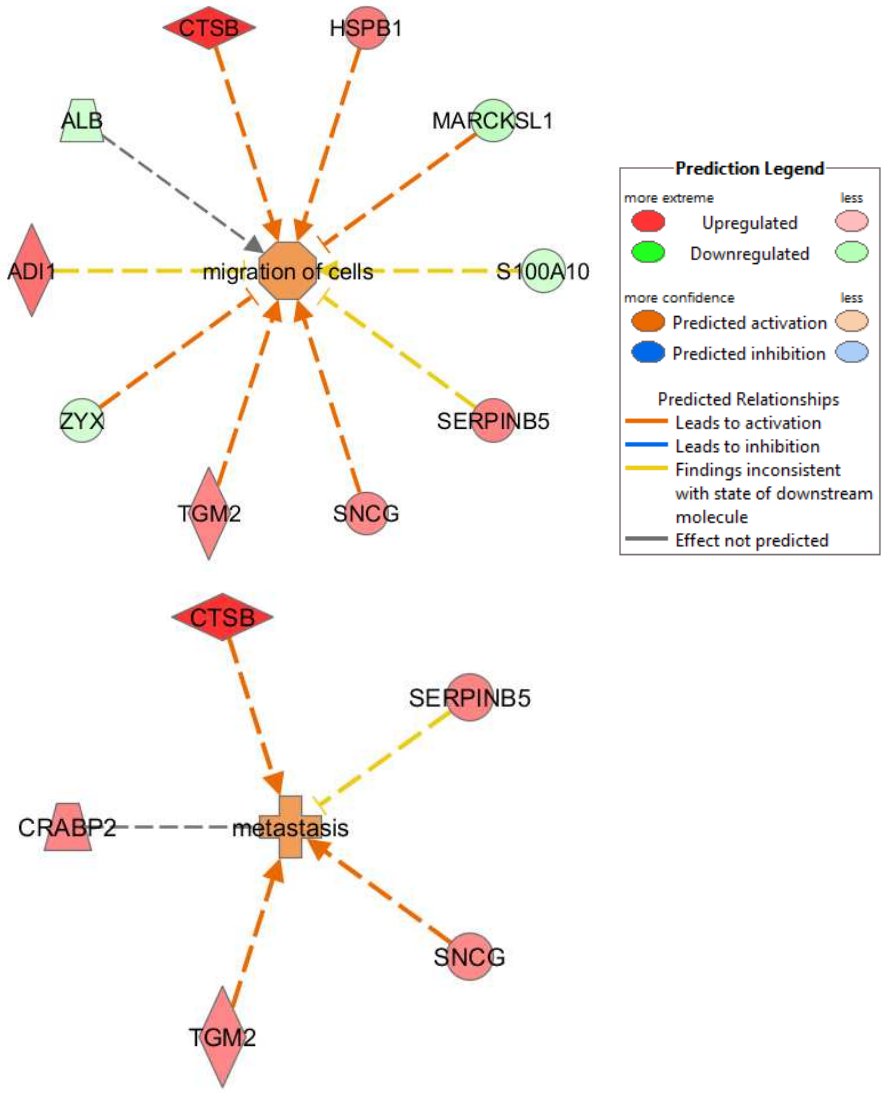


Figure 10. Diagrams showing the downstream effects of some of the proteins found to be common to all three clones. For a key to what the different shapes represent refer to supplementary Figure 4.

4 Discussion

The purpose of chapter 4 was to determine whether the characteristics shown by the clones that lead to an increase in survival, migratory behaviour and chemoresistance (Chapters 1-3) could be explained by changes seen in their proteome. Overall a number of proteins known to be related to metabolism, increased invasive phenotype, metastatic behaviour, drug resistance, stemness and evasion of cell death were found to be altered in all three clones. Showing that these cells, which managed to survive within the necrotic core of MCTS, may have done so as a result of their differential proteomic profile compared to the parental cell line. By characterising the changes seen in the clones new targets for therapeutic intervention could be identified. This discussion aims to highlight the key results obtained in the context of target identification. To start with however methodological aspects will be discussed as these inform the design of subsequent experiments.

4.1 Experimental Procedures: Acetone Precipitation

During the preparation of the protein samples it was necessary to precipitate the protein using acetone, a commonly used protein concentration technique in iTRAQ proteomics. To ensure that enough protein was being precipitated to continue the experiment and that the same amounts of protein were carried forward in each sample the protein concentration was measured before and after acetone treatment. Over half of the total protein in each sample was lost during acetone precipitation (Appendix, Table 4). This reduction in protein yield explains the low number of proteins detected in the initial 8-plex iTRAQ experiment. Because so much protein is lost during the precipitation reaction, it was necessary to start the experiment with an increased amount of sample to account for the loss. Furthermore the protein losses were not the same in each sample therefore it was necessary to measure the protein concentration after acetone precipitation to ensure that for each sample the same amount of protein was being

labelled. Many methodologies do not call for protein measurements post acetone precipitation but to ensure that an equal amount of protein is being labelled from each sample the results of this study found that it was in fact necessary. Omission of this step would therefore invalidate the quantitative nature of the results.

4.2 Necrotic Core Clones

To investigate the proteomic differences seen in the clones compared to the parental cell line; the proteome of three of the clones was investigated alongside the parental cell line. While a proteomics analysis can produced a large volume of data, many different proteins involved in the various process previously investigated such as migration, metastasis, chemoresistance, evasion of cell death, and stem cell resistance pathways can be identified using gene ontology software. In the previous Chapters, the phenotypic changes seen in the clones compared to the parental cell line were described and analysed. The clones were originally found to be slower growing (Chapter 1, Figure 21), have increased migration abilities (Chapter 2, Figure 10), increased chemoresistance (Chapter 3, Table 5) and stem-like and EMT characteristics (Chapter 2, Figure 8 and Figure 11). Many of these characteristics were found to correlate with the proteomic analysis, details of which are discussed in the subsequent sections.

4.2.1 Stem Cell Markers

The identification of cancer stem cells is a contentious issue as discussed in the Introduction. In order to confidently define a cancer stem cell a combination of different markers and assays are required. Several known colorectal stem cell markers were found in the necrotic core of HT-29 MTCS, where the clones were derived from (Chapter 1, Figure 7). Analysis of stem cell functionality through the sphere formation assay found that NCC5 had an increased sphere formation capability Chapter 2, Figure 8). Proteomic analysis found up regulation of a further colorectal cancer stem cell

marker Aldehyde Dehydrogenase (ALDH1) in Clone 4 and 5. ALDH1 is a cytosolic enzyme which oxidises aldehydes to their corresponding carboxylic acids.[10] Expression of Aldehyde Dehydrogenase has been used extensively to identify colorectal cancer stem cells.[11,12] $ALDH1^+$ cells have been found in the base of crypt in the colon epithelium, and during tumourigenesis these cells have been seen to move up through the crypt and increase in number. When implanted into immunocompromised mice, they formed a xenograft whereas $ALDH1^-$ cells were unable to.[13] Long before ALDH1's association with stem cells was discovered, ALDH1 was found to be crucial to drug resistance capabilities in the case of certain drugs such as cyclophosphamide as the drug is a substrate for the cytosolic enzyme.[14, 15] Later on it was implicated in the increased chemoresistance qualities seen in stem cells.[16]

The identification of increased ALDH1 levels in NCC4 and NCC5 further validates the data discussed in Chapter 2 regarding the functional stem cell like properties of the Necrotic Core Clones. Due to the contention surrounding the identification of stem cells discussed previously, different stem cell markers are used for different subtypes of cancer, and marker expression is not stable but can change depending on the environment the cell is placed in. Therefore combinations of marker expression and functional assays such as sphere formation and xenotransplanatation are required.[17] These clones while showing certain marker expression and sphere formation capabilities along with the ability to form tumours *in vivo* certainly have increased stem-like capabilities, though further characterisation would be required before the title 'stem cell' could be used with confidence.

4.2.2 Metastasis and Invasion

All three clones were found to have increased levels of proteins involved in invasion and metastasis of cancer cells. SPINK1 a serine protease inhibitor Kazal type 1 is also

known as Tumour-associated Trypsin Inhibitor or Pancreatic Secretory Inhibitor (PSTI) since this peptide is secreted together with trypsinogen by pancreatic acinar cells and in the gastrointestinal and urinary tract. SPINK1was one of the proteins found to be up regulated in NCC4, the most migratory clone (Table 10) and is known to be associated with metastasis in cancer cells. In particular it was found in the medium of HT-29 cells and was found to increase invasion.[18] Increased SPINK1 expression in liver metastasis has been reported and this was associated with poor prognosis in colorectal cancer.[19] B-CAM, a glycoprotein which functions as a laminin receptor and is believed to play a role in cellular adhesion,[20] was discovered to be up regulated in both NCC4 and NCC5 but not in the least migratory clone, NCC1. This protein has been found to be involved in increased migration in epithelial skin cancers.[21] Cathepsin B a thiol protease was also up-regulated and again this protein is associated with an increased metastatic and invasive potential in cancer cells.[22] Its expression has been found to be particularly up-regulated at the invasive edge of tumours.[23] Cathepsin B plays a role in enabling the degradation of the ECM, through proteolytic cleavage further supporting its role in invasion.[24]

Metastasis associated protein 2 (MTA2) is part of the metastasis associated family of proteins. It is found expressed in solid tumours, where its expression is associated with increased stage of cancer and metastasis. Furthermore, knock down of the MTA2 gene with shRNA in gastric cancer cells caused an *in vitro* reduction in migration and invasion.[25] MTA2 has chromatin remodelling capabilities and is known to be an integral part of the histone deacetylase complexes (HDAC).[26, 27] The HDAC1-containing complex, known as PID, which contains MTA2 is involved in mediating the deacetylation of p53. PID specifically interacts with p53, and reduces the levels of the acetylated protein thereby repressing p53's transciptional activity.[28] MTA2 was found at increased levels in all three of the clones.

Quiescin sulfhydryl oxidase 2 (QSOX2) is an enzyme involved in the oxidation of sulfhydryl groups. QSOX2 is known to be up-regulated under hypoxic conditions and its increased expression has been found to correlate with metastatic potential in pancreatic cancer.[29] Its pro-invasive role has been shown to be mediated through its ability to activate MMP-9.[30,31] In the clones it's expression was found to be up-regulated in all three of the clones.

In summary there were a number of proteins involved in the migration, metastasis and invasion of cancer which were found to be up-regulated in the clones. Further investigation into the reliance of the cells upon these proteins could lead to the discovery of therapeutic targets for preventing or curbing metastasis.

4.2.3 Chemoresistance

The chemoresistance shown by several of the clones discussed in Chapter 3 is a key issue to investigate. Once the cells which manage to survive the low nutrient oxygen conditions found in necrotic tissue are once again able to access oxygen and nutrients and return to a proliferative state any acquired or innate chemoresistant properties could have a significant bearing upon the outcome of therapy. By elucidating some of the mechanisms involved in the increased chemoresistant phenotype this could lead to further targets for chemotherapeutic intervention.

Two pathways, of particular relevance in NCC1 were drug metabolism and DNA repair. These were important findings as NCC1 was found to be the most chemoresistant clone (Table 10). Individual proteins found at higher levels included zeta-crystallin a NAPDH:quinone oxidoreductase.[32] Also increased was Glutathione S-transferase a detoxification enzyme involved in the phase two metabolism of many different types of drug including Chlorambucil, Melphalan, Nitrogen mustard, Phosphoramide mustard, Acrolein, Carmustine, Hydroxyalkenals, Ethacrynic acid, and various steroids.[33]

Importantly Glutathione S- transferase is also thought to be involved in Doxorubicin resistance in colorectal cancer.[34, 35] When cell lines were transfected to overexpress GST their resistance to Doxorubicin was increased.[36, 37] This could indeed explain NCC1's decreased response to Doxorubicin in the MTT assay described in Chapter 3 (Figure 1).

QSOX2, an oxidoreductase also implicated in invasion, metastasis and extracellular matrix formation, was found up regulated in all three clones.[38] It is a protein capable of generating disulfide bonds which enables it to oxidise a wide range of thiol compounds.[39] It has been found to regulate the response of neuroblastoma cells to IFN-gamma radiation.[40]

4.2.4 Cell Death and Survival

DNAJC15 also known as MCJ (methylation-controlled J protein) is a protein located inside the mitochondria. Its function involves negatively regulating mitochondrial membrane potential and the production of ATP. This protein was found up regulated in all three clones and can function as a protective mechanism for tumour cells particularly those under metabolic or chemotherapeutic stress. By down-regulating the activity of the mitochondria the cells are more resistant to mitochondria mediated cell death (namely apoptosis stimulated by the intrinsic pathway).[41, 42, 43]

Other proteins in the cell death and survival pathway that were found to have altered expression in the clones include Heat shock protein beta-1 (HSPB1). HSPB1 has many functions including thermo tolerance, chaperone abilities, regulation of cell differentiation and development and most importantly, inhibition of apoptosis.[44, 45] HSPB1 is also involved in activation of the proteasome and can help increase the degradation of damaged proteins and organelles by binding to ubiquitinated proteins. Furthermore HSPB1 also functions to activate the NF-κβ pathway which subsequently can have effects upon cellular proliferation and inflammation and stress responses.[46] It is this modulation of the NF-κβ pathway along with HSPB1's ability to modulate

reactive oxygen species and increase glutathione levels that is thought to account for its cell survival and anti-apoptotic effects.

4.2.5 Metabolism

Other pathways which were altered in the clones included pathways involved in metabolism and cell survival. As the clones were formed from the cells which managed to survive the harsh environment in the necrotic core, it is likely that alterations would occur in their metabolism or cell survival pathways which enabled them to survive. The metabolism of lipids was found to be changed in all three clones. Up-regulation of proteins such as alanine aminotransferase 2 (ALAT2), a protein involved in glutaminolysis was seen. ALAT2 is responsible for catalysing the reversible transfer of amino groups from L-alanine to α-ketoglutarate, a key substrate for prolyl hydroxylases and HIF1α regulation,[47, 48] to produce pyruvate and L-glutamate. [49] This protein amongst others is found up regulated in a number of different cancers and the inhibition of which has been shown to decrease cell survival[50] and inhibit cellular proliferation and growth.[51, 52] Palmitoyl-protein thioesterase 1 (PPT1) is another protein found up-regulated in the clones which has been found to have protective effects. PPT1 is responsible for removal of palmitic acid (a process called depalmitoylation) from various signalling proteins known to be deregulated in cancer such as Src- and Ras-related proteins. The process of depalmitoylation is required before such proteins can degraded by lysosomal proteases.[53] This protein was found to be important for cell survival in cancer cells, it's over expression has been shown to have a protective effect for cells against cell death whereas its inhibition has shown to induce apoptosis.[54]

4.2.6 Autophagy

Several proteins known to be involved in autophagy were found to have altered expression in the necrotic core clones. These include Cathepsin B, Sequestosome-1 and

Tissue tranglutaminase 2. Cathepsin B is a proteolytic enzyme involved in many different processes associated with cancer including invasion and metastasis and autophagy. Increased expression has been shown to increase the invasive and metastatic potential of the cancer cells.[55] Cathepsin B is also known to have a role in autophagy, the degradation pathway which has been found to be up regulated in the necrotic core of MCTS.[56] Increased levels of Cathepsin B have been shown to protect cells from cell death during times of nutrient deprivation.[57] Increased expression of this protein was found in all three clones and could go some way towards explaining their survival within the necrotic core.

Sequestosome-1 was found to be down regulated in the clones, this protein also known as p62 is involved in many different signalling pathways, including autophagy, activation of Nuclear Factor-$_K$B, oxidative signalling,[58] the extrinsic apoptosis pathway and tumourigenesis.[59] Increased tumour autophagy has been shown to decrease protein levels of Sequestosome-1 which is an autophagy substrate itself as it acts as a molecular adaptor between the autophagic machinery and its substrates for degradation.[6061] It is also involved in the extrinsic apoptotic pathway through its role in the aggregation of caspase-8.[62]

Tissue Transglutaminase 2 (TGM2) is another protein which has links to autophagy that was found to be up regulated in the clones. TGM2 is a key component in the maturation of the autophagolysosome. [63] Increased levels of the proteins have been found in numerous different cancer types, and is associated with increased tumour aggression, poor prognosis, invasion and metastasis and drug resistance. [64] Its drug resistance abilities are thought to be mediated through its modulation of survival pathways, apopotosis, ECM formation, EMT and autophagy.[65] Recently TGM2 has been found to induce EMT and stem like characteristics in epithelial cells.[66] This supports much of the

proteomic data presented here as many stem cell and migratory related proteins have been found to be altered in the clones.

Table 10. Table of characteristics applying to the Necrotic Core Clones which have been measured *in vitro*. Green indicates up-regulated or increased activity, and red indicates a decrease. Characteristics were determined using the following assays: MTT assay, wound healing assay, proliferation assay and the sphere formation assay. All changes shown were statically significant ($p<0.05$)

Clone	Chemosensitivity	Migration	Proliferation	Anchorage Independent Growth
Necrotic Core Clone1				
Necrotic Core Clone 2				
Necrotic Core Clone 3				
Necrotic Core Clone 4				
Necrotic Core Clone 5				

4.3 Conclusion

The proteomic analysis of the necrotic core clones in comparison to the parental cell line has shown that there are obvious differences in the expression of proteins. Many of these differentially expressed proteins have roles in a number of different processes which were found phenotypically to be altered in the clones. For example the clones, in particular NCC4, were found to have increased migratory behaviour and numerous different proteins found to be involved in migration, invasion and metastasis and the

EMT were up regulated in the clones compared to the parental cell line. NCC1 was found to be more chemoresistant to Doxorubicin and 5-FU *in vitro*, when the proteome of NCC1 was examined it was found that there was up regulation of Glutathione S-transferase an enzyme that has in fact been linked to Doxorubicin resistance in colorectal cancer. Moreover NCC5 was found to have a more stem like phenotype *in vitro* compared to the parental cell line, the proteomic analysis found increased expression of Aldehyde dehydrogenase (ALDH1A1) a colorectal stem cell marker in NCC5 as well as NCC4.

By looking at the changed proteins which were common to all three clones, clues as to the possible mechanisms by which these cells managed to survive within the necrotic core of the HT-29 MCTS can be elucidated. The top molecular function identified by the gene ontology software was cell death and survival, and involved 22 out of the 49 changed proteins. This was expected as the one main difference between the parental cell line and the clones is that the clones are capable of escaping cell death under conditions which kill the parental cells. Proteins involved in the regulation of the cell cycle were also found changed in of the clones; this was not unexpected for two main reasons. The proliferation status of the clones was found to be significantly lower than the parental cell line when returned to normal culture conditions, a change which seemed to be stable in the cells, and when the proliferation status of the MCTS cells *in situ* was examined the living cells within the necrotic core were not found to be actively proliferating.

Metabolism was a further function which was found to be altered in the clones, again this was not unexpected because in order to survive the low nutrient conditions of the necrotic core, cells would have to have undergone some sort of metabolic adaptation. Lipid metabolism and various proteins involved in autophagy were shown to be altered.

In particular the expression of autophagy related proteins was indicative of an increased level of autophagic flux within the cells. These results are consistent with the observation that autophagy was found to be actively occurring in the necrotic core when the MCTS were examined immunohistochemically, Furthermore this suggests that these cells have a higher level of basal autophagy and therefore are better equipped to survive poor nutrient conditions. This also proves that the increased levels of autophagy seen in the necrotic core was not a transient change only seen while the cells were within the necrotic core but seems to be a stable change.

One of the most valuable things learnt from the proteomic analysis of the clones was that the changes induced or selected for by the necrotic environment seem to have been memorised and remain as stable changes even when the cells are returned to favourable conditions. An important question this raises is whether the necrotic core induces these changes in the cells or merely selects for cells which already possess the ability to survive the hostile conditions. Regardless of the answer if this scenario was applied to a clinical situation, the end result is still an enriched population of cells which have increased metastatic potential, survival ability and stem like characteristics. By their very nature, they may be resistant to most chemotherapies and can therefore contribute to poor clinical outcome.

4.3.1 Potential Targets

The analysis of the proteome of the necrotic core clones has given rise to a number of possible targets, some of which have been targeted previously. One of these possible targets is Cathepsin B found up regulated in the clones. Inhibitors of this protein have been tested both *in vitro, in vivo* and in the clinic and while they have been found to be effective in reducing tumour cell motility and invasiveness they have had issues with off target effects and toxicities.[67] Another autophagy related target is TGM2, inhibition of

this protein has been found to reverse drug resistance in multiple cancer types including glioblastoma[68] and breast.[69] Specifically in breast cancer it was found to be responsible for Doxorubicin resistance, similar to NCC1. Therefore inhibition of this protein in the necrotic core clones would be very interesting to look into. PPT1 inhibitors have also been tested in cancer cells. In neuroblastoma the inhibition of PPT1 was found to selectively kill the cells, although this work has yet to progress to *in vivo* or clinical testing PPT1 could be a valid target. Gluathione S-transferease which was found up-regulated in NCC1 the most chemoresstant clone could also be a possible target to sensitise cells to Doxorubicin treatment. ALAT2 which is involved in cellular metabolism has also been suggested as a target for cancer therapy. Inhibition of the protein has been found to reduce cancer cell proliferation growth and survival. Furthermore *in vivo* research has found that ALAT2 inhibition exerts this effect by stimulating mitochondrial activity, cancer growth is therefore impaired as ALAT2 inhibition counteracts the Warburg effect by up regulating mitochondrial metabolism.[70] MTA2 is another target which has been tested *in vivo*. Inhibition of this metastasis associated protein was found to reduce xenograft growth and lung metastasis in immunocompromised mice. Inhibiting this protein therefore could have effects on not only the primary tumour but this supposedly would also inhibit those cells with metastatic potential which would otherwise be able to metastasise and from new metastatic deposits. However all these targets need to be validated in colorectal cancer. Though the fact that work has been carried out previously in other tumour types and has shown good results is beneficial and provides further evidence that they are valid cancer targets.

5 References

[1] Human Genome Project. 2014, U.S. Department of Energy Office of Science, Office of Biological and Environmental Research, accessed 3rd July 2014, <http://web.ornl.gov/sci/techresources/Human_Genome/index.shtml>

[2] Baak JP, Janssen EA, Soreide K, Heikkilae R. Genomics and proteomics--the way forward. *Ann Oncol*. 2005;16 Suppl 2:ii30-44.

[3] Fine JM, Creyssel R. Starch gel electrophoresis studies on abnormal proteins in myeloma and macroglobulinaemia. *Nature*. 1959;183(4658):392.

[4] Applied Biosystems iTRAQ™ Reagents: Chemistry Reference Guide. Applied Biosystems, 2004.

[5] Fu J, Yang QY, Sai K, Chen FR, Pang JC, Ng HK, Kwan AL, Chen ZP. TGM2 inhibition attenuates ID1 expression in CD44-high glioma-initiating cells. *Neuro Oncol*. 2013;15(10):1353-65.

[6] Zhou C, Ji J, Cai Q, Shi M, Chen X, Yu Y, Liu B, Zhu Z, Zhang J. MTA2 promotes gastric cancer cells invasion and is transcriptionally regulated by Sp1. *Mol Cancer*. 2013;12(1):102.

[7] Thornton TM, Rincon M. Non-classical p38 map kinase functions: cell cycle checkpoints and survival. *Int J Biol Sci*. 2009;5(1):44-51.

[8] Ramsay EE, Hogg PJ, Dilda PJ. Mitochondrial metabolism inhibitors for cancer therapy. *Pharm Res*. 2011;28(11):2731-44.

[9] Hatle KM, Gummadidala P, Navasa N, Bernardo E, Dodge J, Silverstrim B, Fortner K, Burg E, Suratt BT, Hammer J, Radermacher M, Taatjes DJ, Thornton T, Anguita J, Rincon M. MCJ/DnaJC15, an endogenous mitochondrial repressor of the respiratory chain that controls metabolic alterations. *Mol Cell Biol*. 2013;33(11):2302-14.

[10] Ikawa M, Impraim CC, Wang G, Yoshida A. Isolation and characterization of aldehyde dehydrogenase isozymes from usual and atypical human livers. *J Biol Chem*. 1983;258(10):6282-7.

[11] Huang EH, Hynes MJ, Zhang T, Ginestier C, Dontu G, Appelman H, Fields JZ, Wicha MS, Boman BM. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. *Cancer Res*. 2009;69(8):3382-9.

[12]Armstrong L, Stojkovic M, Dimmick I, Ahmad S, Stojkovic P, Hole N, Lako M. Phenotypic characterization of murine primitive hematopoietic progenitor cells isolated on basis of aldehyde dehydrogenase activity. *Stem Cells*. 2004;22(7):1142-51.

[13] Huang EH, Hynes MJ, Zhang T, Ginestier C, Dontu G, Appelman H, Fields JZ, Wicha MS, Boman BM. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. *Cancer Res*. 2009;69(8):3382-9.

[14] Magni M, Shammah S, Schiró R, Mellado W, Dalla-Favera R, Gianni AM. Induction of cyclophosphamide-resistance by aldehyde-dehydrogenase gene transfer. *Blood*. 1996;87(3):1097-103.

[15] Hilton J. Role of aldehyde dehydrogenase in cyclophosphamide-resistant L1210 leukemia. *Cancer Res.* 1984;44(11):5156-60.

[16] Abdullah LN, Chow EK. Mechanisms of chemoresistance in cancer stem cells. *Clin Transl Med.* 2013;2(1):3.

[17] Yeung TM, Gandhi SC, Wilding JL, Muschel R, Bodmer WF. Cancer stem cells from colorectal cancer-derived cell lines. *Proc Natl Acad Sci U S A.* 2010;107(8):3722-7.

[18] Gouyer V, Fontaine D, Dumont P, de Wever O, Fontayne-Devaud H et al. Autocrine induction of invasion and metastasis by tumor-associated trypsin inhibitor in human colon cancer cells. *Oncogene.* 2008; 27: 4024–33.

[19] Gaber A, Johansson M, Stenman UH, Hotakainen K, Ponten F et al. High expression of tumour-associated trypsin inhibitor correlates with liver metastasis and poor prognosis in colorectal cancer. *Br J Cancer*. 2009; 100: 1540–8.

[20] Schön M, Klein CE, Hogenkamp V, Kaufmann R, Wienrich BG, Schön MP. Basal-cell adhesion molecule (B-CAM) is induced in epithelial skin tumors and inflammatory epidermis, and is expressed at cell-cell and cell-substrate contact sites. *J Invest Dermatol*. 2000;115(6):1047-53.

[21] Drewniok C, Wienrich BG, Schön M, Ulrich J, Zen Q, Telen MJ, Hartig RJ, Wieland I, Gollnick H, Schön MP. Molecular interactions of B-CAM (basal-cell adhesion molecule) and laminin in epithelial skin cancer. *Arch Dermatol Res*. 2004;296(2):59-66.

[22] Gopinathan A, Denicola GM, Frese KK, Cook N, Karreth FA, Mayerle J, Lerch MM, Reinheckel T, Tuveson DA. Cathepsin B promotes the progression of pancreatic ductal adenocarcinoma in mice. *Gut.* 2012;61(6):877-84.

[23] Berquin IM, Sloane BF. Cathepsin B expression in human tumors. *Adv Exp Med Biol*. 1996;389:281-94.

[24] Mai J, Finley RL Jr, Waisman DM, Sloane BF. Human procathepsin B interacts with the annexin II tetramer on the surface of tumor cells. *J Biol Chem*. 2000;275(17):12806-12.

[25] Zhou C, Ji J, Cai Q, Shi M, Chen X, Yu Y, Liu B, Zhu Z, Zhang J. MTA2 promotes gastric cancer cells invasion and is transcriptionally regulated by Sp1. *Mol Cancer*. 2013;12(1):102.

[26] Zhang Y, LeRoy G, Seelig HP, Lane WS, Reinberg D. The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. *Cell*. 1998;95(2):279-89.

[27] Xue Y, Wong J, Moreno GT, Young MK, Côté J, Wang W. NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. *Mol Cell.* 1998;2(6):851-61.

[28] Luo J, Su F, Chen D, Shiloh A, Gu W. Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature*. 2000;408(6810):377-81.

[29] Katchman BA, Antwi K, Hostetter G, Demeure MJ, Watanabe A, Decker GA, Miller LJ, Von Hoff DD, Lake DF. Quiescin sulfhydryl oxidase 1 promotes invasion of pancreatic tumor cells mediated by matrix metalloproteinases. *Mol Cancer Res*. 2011;15:1621–1631.

[30] Katchman BA, Ocal IT, Cunliffe HE, Chang YH, Hostetter G, Watanabe A, Lobello J, Lake DF. Expression of quiescin sulfhydryl oxidase 1 is associated with a highly invasive phenotype and correlates with a poor prognosis in Luminal B breast cancer. *Breast Cancer Res*. 2013;15(2):R28.

[31] Shi CY, Fan Y, Liu B, Lou WH. HIF1 Contributes to Hypoxia-Induced Pancreatic Cancer Cells Invasion via Promoting QSOX1 Expression. *Cell Physiol Biochem*. 2013;32(3):561-8.

[32] Rao PV, Krishna CM, Zigler JS Jr. Identification and characterization of the enzymatic activity of zeta-crystallin from guinea pig lens. A novel NADPH:quinone oxidoreductase. *J Biol Chem*. 1992;267(1):96-102.

[33] Townsend DM, Tew KD. The role of glutathione-S-transferase in anti-cancer drug resistance. *Oncogene*. 2003;22(47):7369-75.

[34] Goto S, Ihara Y, Urata Y, Izumi S, Abe K, Koji T, Kondo T. Doxorubicin-induced DNA intercalation and scavenging by nuclear glutathione S-transferase pi. *FASEB J*. 2001;15(14):2702-14.

[35] Beaumont PO, Moore MJ, Ahmad K, Payne MM, Lee C, Riddick DS. Role of glutathione S-transferases in the resistance of human colon cancer cell lines to doxorubicin. *Cancer Res*. 1998;58(5):947-55.

[36] He NG, Singhal SS, Srivastava SK, Zimniak P, Awasthi YC, Awasthi S. Transfection of a 4-hydroxynonenal metabolizing glutathione S-transferase isozyme, mouse GSTA4-4, confers doxorubicin resistance to Chinese hamster ovary cells. *Arch Biochem Biophys*. 1996;333(1):214-20.

[37] Nakagawa K, Saijo N, Tsuchida S, Sakai M, Tsunokawa Y, Yokota J, Muramatsu M, Sato K, Terada M, Tew KD. Glutathione-S-transferase pi as a determinant of drug resistance in transfectant cell lines. J Biol Chem. 1990;265(8):4296-301.

[38] Coppock DL, Thorpe C. Multidomain flavin-dependent sulfhydryl oxidases. *Antioxid Redox Signal*. 2006;8(3-4):300-11.

[39] Kodali VK, Thorpe C. Oxidative protein folding and the Quiescin-sulfhydryl oxidase family of flavoproteins. *Antioxid Redox Signal*. 2010;13(8):1217-30.

[40] Wittke I, Wiedemeyer R, Pillmann A, Savelyeva L, Westermann F, Schwab M. Neuroblastoma-derived sulfhydryl oxidase, a new member of the sulfhydryl oxidase/Quiescin6 family, regulates sensitization to interferon gamma-induced cell death in human neuroblastoma cells. *Cancer Res*. 2003;63(22):7742-52.

[41] Gogvadze V, Zhivotovsky B, Orrenius S. The Warburg effect and mitochondrial stability in cancer cells. *Mol Aspects Med.* 2010;31(1):60-74.

[42] Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria in cancer cells: what is so special about them? *Trends Cell Bio*l. 2008;18(4):165-73.

[43] Hatle KM, Gummadidala P, Navasa N, Bernardo E, Dodge J, Silverstrim B, Fortner K, Burg E, Suratt BT, Hammer J, Radermacher M, Taatjes DJ, Thornton T, Anguita J, Rincon M. MCJ/DnaJC15, an endogenous mitochondrial repressor of the respiratory chain that controls metabolic alterations. *Mol Cell Biol*. 2013;33(11):2302-14.

[44] Arrigo AP. In search of the molecular mechanism by which small stress proteins counteract apoptosis during cellular differentiation. *J Cell Biochem*. 2005;94(2):241-6.

[45] Sarto C, Binz PA, Mocarelli P. Heat shock proteins in human cancer. *Electrophoresis*. 2000;21(6):1218-26.

[46] Parcellier A, Schmitt E, Gurbuxani S, Seigneurin-Berny D, Pance A, Chantôme A, Plenchette S, Khochbin S, Solary E, Garrido C. HSP27 is a ubiquitin-binding protein involved in I-kappaBalpha proteasomal degradation. *Mol Cell Biol*. 2003;23(16):5790-802.

[47] Ward PS, Thompson CB. Signaling in control of cell growth and metabolism. *Cold Spring Harb Perspect Biol*. 2012;4(7):a006783.

[48] Tennant DA, Gottlieb E. HIF prolyl hydroxylase-3 mediates alpha-ketoglutarate-induced apoptosis and tumor suppression. *J Mol Med (Berl).* 2010;88(8):839-49.

[49] Wise DR, Thompson CB. Glutamine addiction: a new therapeutic target in cancer. *Trends Biochem Sci*. 2010;35(8):427-33.

[50] Qin JZ, Xin H, Nickoloff BJ. Targeting glutamine metabolism sensitizes melanoma cells to TRAIL-induced death. *Biochem Biophys Res Commun*. 2010;398(1):146-52.

[51] Thornburg JM, Nelson KK, Clem BF, Lane AN, Arumugam S, Simmons A, Eaton JW, Telang S, Chesney J. Targeting aspartate aminotransferase in breast cancer. *Breast Cancer Res*. 2008;10(5):R84.

[52] Wise DR1, DeBerardinis RJ, Mancuso A, Sayed N, Zhang XY, Pfeiffer HK, Nissim I, Daikhin E, Yudkoff M, McMahon SB, Thompson CB. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc Natl Acad Sci U S A*. 2008;105(48):18782-7.

[53] Zhang Z, Lee YC, Kim SJ, Choi MS, Tsai PC, Xu Y, Xiao YJ, Zhang P, Heffer A, Mukherjee AB. Palmitoyl-protein thioesterase-1 deficiency mediates the activation of the unfolded protein response and neuronal apoptosis in INCL. *Hum Mol Genet*. 2006;15(2):337-46.

[54] S. Cho, G. Dawson. Palmitoyl protein thioesterase 1 protects against apoptosis mediated by Ras–Akt–caspase pathway in neuroblastoma cells. *J. Neurochem*. 2000;74:1478–1488.

[55] Gopinathan A1, Denicola GM, Frese KK, Cook N, Karreth FA, Mayerle J, Lerch MM, Reinheckel T, Tuveson DA. Cathepsin B promotes the progression of pancreatic ductal adenocarcinoma in mice. *Gut.* 2012;61(6):877-84.

[56] Tatti M, Motta M, Di Bartolomeo S, Scarpa S, Cianfanelli V, Cecconi F, Salvioli R. Reduced cathepsins B and D cause impaired autophagic degradation that can be almost completely restored by overexpression of these two proteases in Sap C-deficient fibroblasts. *Hum Mol Genet*. 2012;21(23):5159-73.

[57] Uchiyama Y. Autophagic cell death and its execution by lysosomal cathepsins. *Arch Histol Cytol*. 2001;64(3):233-46.

[58] Puissant A, Fenouille N, Auberger P. When autophagy meets cancer through p62/SQSTM1. *Am J Cancer Res*. 2012;2(4):397-413.

[59] Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, Bray K, Reddy A, Bhanot G, Gelinas C, Dipaola RS, Karantza-Wadsworth V, White E. Autophagy suppresses tumorigenesis through elimination of p62. *Cell*. 2009;137(6):1062-75.

[60] Puissant A, Fenouille N, Auberger P. When autophagy meets cancer through p62/SQSTM1. *Am J Cancer Res*. 2012;2(4):397-413.

[61] Groulx JF, Khalfaoui T, Benoit YD, Bernatchez G, Carrier JC, Basora N, Beaulieu JF. Autophagy is active in normal colon mucosa. *Autophagy*. 2012;8(6):893-902.

[62] Jin Z, Li Y, Pitti R, Lawrence D, Pham VC, Lill JR, Ashkenazi A. Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. *Cell*. 2009;137(4):721-35.

[63] D'Eletto M, Farrace MG, Rossin F, Strappazzon F, Giacomo GD, Cecconi F, Melino G, Sepe S, Moreno S, Fimia GM, Falasca L, Nardacci R, Piacentini M. Type 2 transglutaminase is involved in the autophagy-dependent clearance of ubiquitinated proteins. *Cell Death Differ*. 2012 ;19(7):1228-38.

[64] Agnihotri N, Kumar S, Mehta K. Tissue transglutaminase as a central mediator in inflammation-induced progression of breast cancer. *Breast Cancer Res*. 2013;15(1):202.

[65] Budillon A, Carbone C, Di Gennaro E. Tissue transglutaminase: a new target to reverse cancer drug resistance. *Amino Acids*. 2013;44(1):63-72.

[66] Kumar S, Mehta K. Tissue transglutaminase, inflammation, and cancer: how intimate is the relationship? *Amino Acids*. 2013;44(1):81-8.

[67] Frlan R, Gobec S. Inhibitors of cathepsin B. *Curr Med Chem*. 2006;13(19):2309-27.

[68] Yuan L, Choi K, Khosla C, Zheng X, Higashikubo R, Chicoine MR, Rich KM. Tissue transglutaminase 2 inhibition promotes cell death and chemosensitivity in glioblastomas. *Mol Cancer Ther*. 2005;4(9):1293-302.

[69] Kim DS, Park SS, Nam BH, Kim IH, Kim SY. Reversal of drug resistance in breast cancer cells by transglutaminase 2 inhibition and nuclear factor-kappaB inactivation. *Cancer Res*. 20065;66(22):10936-43.

[70] Beuster G, Zarse K, Kaleta C, Thierbach R, Kiehntopf M, Steinberg P, Schuster S, Ristow M. Inhibition of alanine aminotransferase in silico and in vivo promotes mitochondrial metabolism to impair malignant growth. *J Biol Chem*. 2011;286(25):22323-30.

Discussion

1 Discussion

Tumour necrosis has been linked to poor patient prognosis since the early 1990s,[1] yet no conclusive mechanism has been discovered to explain the relationship. Many hypotheses have been proposed yet scarce research has been carried out to support them, possibly due to necrotic tissue not being considered a necessary target for chemotherapeutic intervention. To date the proposed hypotheses include, the presence of necrotic tissue correlates with hypoxia which is linked to decreased radiosensitivity,[2] and chemosensitivity.[3] Tumour necrosis occurs in fast growing aggressive tumours which quickly outgrow their blood supply and therefore necrosis occurs as a result of the tumour cells' aggressive nature.[4, 5] And finally the idea that the process of necrotic cell death itself stimulates inflammation leading to an environment conducive to mutagenesis, cell proliferation and subsequent immunosuppression.[6] The discovery of living cells within the necrotic core of MCTS highlights the importance of understanding the link between tumour necrosis and patient prognosis, especially as tumour necrosis is a common feature of many different types of solid tumour.

The aims of this thesis were to understand how and why tumour necrosis was linked to poor prognosis by focussing on the necrotic core of multicellular tumour spheroids and the cells which reside there. The major finding of this thesis is the identification and isolation of viable cells from the necrotic core, which provides an alternative explanation for why necrosis is associated with poor prognosis. This discussion will evaluate the work done in this thesis to characterise these cells in order to determine their biological and therapeutic significance.

As stated above the primary conclusion to be made from this thesis is that the necrotic core of multicellular tumour spheroids contains living cells. To date necrotic tissue has largely been overlooked when examining the tumour microenvironment. The first

indication of this discovery presented in this thesis occurred when the MCTS were immunohistochemically stained for cleaved capase-3 and a number of cells within the necrotic core showed negative expression. These negatively stained cells were found to be viable when the necrotic core cells were separated from the rest of the cells within the MCTS using flow cytometry and cells were found to grow from this population. When these cells were examined in situ it was discovered they had an altered cellular metabolism, namely they were actively undergoing autophagy and they showed different expression of EMT and stem cell markers compared to the other cells in the MCTS. This then lead to the next important conclusion, not only were there living cells in the necrotic core but these cells were phenotypically distinct from the rest of the cells in the MCTS. The changes in EMT and stem cell markers lead to the hypothesis that these cells could explain why tumours with high levels of necrosis are more aggressive and result in a poor prognosis for the patient. From their expression of EMT and stem cell markers it is possible that they are more migratory and metastatic and have other stem cell properties. Cells such as these have been implicated in the formation of metastases as the tumour cell of origin. Necrotic areas form due to lack of nutrients and oxygen; these surviving cells therefore are more resistant to these conditions as many stem cells have shown to be.[7]

The survival of clones within the necrotic core of MCTS was the first indication that there may be another possible hypothesis to explain the link between tumour necrosis and poor patient prognosis. The survival and possible enrichment of these cells in the tumour population following a spatial and/or temporal decline of oxygen and nutrients may hold the key for how to target these cells for destruction. The formation of necrotic core clones themselves from the living cells within the MCTS necrotic core was an important development in the project. It not only enabled the characterisation of these

cells, specifically their migration and stem cell characteristics through functional assays but allowed the determination as to the state of changes in the cells, were they transient or stable? Transient changes would not explain fully how the viable necrotic core cells were the cause of metastasis if once out of the necrotic tissue they returned to their original state. A stable change on the other hand would allow for the theory that these cells were the cells which cause secondary tumours and relapse. Due to their different properties the cells could be able to survive within the hostile environment, and once conditions became favourable again their metastatic capabilities would allow them to migrate to a new location where stem cell qualities could enable them to form new micro metastases. Whilst these changes were seen to be stable within the clones as functional analyses were carried out several months post isolation from the MCTS, stem like cells are known for their plasticity. Upon attachment to a new location the cell would have to undergo the MET in order to attach and begin to grow. To be more accurate it seems that the necrotic core selects for characteristics that are useful at the current time for the cell and for the future of the tumour.

When analysed functionally, the clones demonstrated their differences compared to the parental cell line. Increased migration seen in the wound healing assay, increased sphere formation capabilities, and increased resistance to chemotherapeutics were characteristics seen in a number of different clones. These observed characteristics were further supported by the proteomic data which showed distinct changes in the proteome of the clones. Altered expression was found in proteins involved in migration, invasion and metastasis, cell survival, cell metabolism and resistance to chemotherapeutics, specifically one of the drugs on which clones response was tested (Doxorubicin). This change in protein expression was seen once the clones had returned to two dimensional cell culture for a substantial amount of time. An interesting discovery from this work

has been that the five necrotic core clones whilst being phenotypically distinct from the parental cell line are also phenotypically distinct from each other. In the various different functional assays they have shown different responses. Cloned stem cell lines from the same tumour as well as from different but histologically similar tumours are known to show a variety of different responses to both chemotherapeutics and radiation.[8,9, 10] Phenotypic stability of these cells is believed to be dependent on the stability of the different environmental selective pressures.[11] The necrotic core clones established in this work mimics the heterogeneous nature of stem-like cell populations found in tumours. When tumour populations have been examined, genetically similar cells were shown to be functionally heterogeneous. Hence proteomic analysis of the cellular heterogeneity might be more accurate in terms of phenotyping the cells accurately. The differing functional capabilities of genetic clones are believed to provide increased survival potential during times of stress and occur as the result of gene expression noise, epigenetic and tumour microenvironment drivers.[12, 13, 14] There is increasing amount of evidence to suggest that diversity generating systems are evolutionarily selected for.[15, 16, 17, 18] Research into single cell prokaryotes found that in homogenous populations a small number of cells showed drug resistance which was independent of genetic change or acquisition of antibiotic resistance containing plasmids. This difference is instead explained by reduced proliferation and entrance into a dormant non-dividing state similar to that of the living cells in the necrotic core.[19]. Colorectal cancer is known to involve a comparatively dormant cell population, which suggests that cancer cells may have taken advantage of this evolutionary conserved process in order to survive in times of hardship. Dormant cells have been extracted from colorectal tumours and found to still retain tumour initiation potential, a phenomenon which was demonstrated using the MCTS. These dormant cells were shown to become

enriched in tumours following the administration of chemotherapeutic drugs. [20] When examined in situ the viable cells surviving in the necrotic core of MCTS were positive for a marker of senescence. What needs to be taken into account is that even cells in homogenous populations don't all respond in a identical matter to stress, this often unstated supposition when using cells in bulk two dimensional monolayer populations means that when cell responses are averaged single cell heterogeneity can be concealed. Separating the MCTS cells into different populations allowed the individual characterisation of the fractions, the results of which highlighted the level of variability seen over very small distances. By investigating the surviving necrotic core fraction for further mechanisms of dormancy and reliance upon certain survival mechanisms, new potential targets could be discovered to specifically target this population.

The metabolic adaptation was investigated with regards to the importance of autophagy for cell survival within the necrotic core. Autophagy was found to be up regulated in the necrotic core cells in situ, and proteins involved in the process of autophagy were later found to still be up-regulated in the proteomic analysis of the clone monolayers. Inhibition of autophagy in MCTS was found to increase cell death at depths previously found to be survivable by the cells. The role of autophagy in cancer is complicated; the autophagy paradox means that autophagy has both pro tumour and tumour suppressive effects,[21] effects which change depending on the stage of tumour formation. In a tumour in the early stages of formation, the inhibition of autophagy can enhance tumourigenesis as it functions to increase cellular stress in the absence of nutrient deprivation (as the tumour has yet to outgrow the local circulation). As cellular stress increases so do the chances of mutations, some of which could be beneficial to the development of the tumour. Conversely in an established tumour, specifically one with areas of necrotic tissue, autophagy can be used as a survival mechanism by the tumour to endure the low

nutrient environment. In the case of established solid tumours therefore, inhibiting autophagy could be a valid target for decreasing the tumour mass and for specifically targeting cells managing to survive in the harshest microenvironments within the tumour. Reducing the number of these metabolically adapted resilient cells such as the necrotic core clones, which have been shown to be more aggressive in vitro, would hopefully reduce the chances of post treatment relapse. Furthermore autophagy have been seen previously to be used by cells as a mechanism of survival when treated with chemotherapeutics,[22] therefore autophagy inhibitions in combination with other cytotoxic drugs could improve the cell kill.

Research in this project has found that autophagic inhibitors have the potential to target and kill hypoxic cells such as those bordering the necrotic core in MCTS. Necrotic cores were found to become enlarged as the critical depth within the MCTS where necrosis is induced was reduced. By inhibiting autophagy in these oxygen and nutrient deprived cells its reduces their survival potential. This demonstrates a way in which to treat non proliferating cancer cells. Inhibiting autophagy has been found to target cells in the hypoxic, nutrient deprived microenvironment in several cancer types including liver[23] and pancreatic cancer.[24] Though as yet no data has been published on the use of autophagy inhibitors to selectively target the tumour microenvironment in colorectal cancer as a single agent.

The colorectal cancer stem cell marker ALDH1 was found to be up regulated in two of the three clones evaluated in the proteomic assay. Specifically in one clone (NCC5) which was shown to have increased anchorage independent growth, a known stem cell characteristic, in the sphere formation assay. And in the other clone (NCC4) which was found to be able to form a similarly heterogeneous tumour in vivo compared to the parental cell line. The general consensus with identifying cancer stem cells is that

cancer stem cells can be definitively defined experimentally by their ability to recapitulate the generation of a continuously growing tumour".[25] Regardless of title however it is clear some of the necrotic core clones have an increased stem-like behaviour compared to the parental cell line. In addition NCC4 was found to be capable of initiating a fully heterogeneous xenografts with a similar structure to the parental cell line. This is regarded as the gold standard of stem cell assays. When the clones were first formed their holoclone morphology was consistent with that of stem-like cells which have the ability to produce differentiated progeny and a high proliferative potential. Clones with this specific compact round morphology are believed to be enriched in carcinoma stem cell populations.[26] Cancer stem cells or tumour initiating cells as they are sometimes known have been found previously in cervical MCTS.[27] The hypoxic necrotic tumour microenvironment found in many solid tumours could be an, albeit harsh, stem cell niche.

The findings from this research have lead to the identification of a number of possible targets as it has discovered certain proteins to be over expressed in the Necrotic Core Clones. These proteins have been found to be involved in some of the mechanisms used by the cancer cells to survive within the necrotic environment. The altered metabolism found in the cells can be targeted, specifically autophagy as discussed previously. The cell's cell cycle status could also be a possible target. The living cells within the necrotic core are not actively proliferating and express certain senescence markers. Senescence is known to be reversible in some cancer cells making these cells potentially dangerous. Targeting senescent cells could therefore be a relatively selective way to target the cells surviving in necrotic areas. Different methods have been suggested to target senescent cancer cells previously due to the observation that senescent tumour cells have detrimental properties which warrant their elimination. Mechanisms of targeting include

utilising the senescent cell's metabolic phenotype which has been described as hyper catabolic. Senescent cells are known to have increased endoplasm reticulum stress and glucose consumption, in combination with the up regulation of unfolded protein response and protein ubiquitination which leads to the increased targeting of proteins for autophagic degradation. Therefore synthetic lethal metabolic targeting has been proposed as a way to specifically eliminate these cells.[28]

Dormancy has previously been implicated in the survival of some populations of cells within a tumour. Cells induced to become quiescent from signals in the microenvironment have been implicated of acting like stem cells to cause re growth of the tumour at later time. [29] Therefore targeting these cells which are normally immune to chemotherapeutics due to their proliferative status has the potential to improve long term treatment outcome. Similarly the differences that stem cells exhibit can be targeted as well. Cancer stem cells themselves are not known to be homogeneous; many different types of cancer stem cell can be present in the same tumour mass each with differing characteristics. This heterogeneous population therefore is not straightforward to target. Not only are cancer stem cells not all alike but they themselves can express different markers and up regulate different signalling pathways depending on their current environment, so a stem cell will not be consistently expressing the same markers for its whole life. However by identifying signalling networks which are deregulated in cancer stem cells or by finding a key molecule on which the stem cells rely on for a critical process such as self-renewal, senescence, chemoresistance, or evasion of cell death, these cells may be targeted. Differentiation therapy can also be used to force the pluripotent cells to terminally differentiate, thereby extinguishing their potential to form new metastatic deposits. Drugs such as retinoic acid have been used to force stem cells to differentiate in leukaemias,[30] squamous cell carcinomas[31] and melanoma,[32] causing

effects ranging from reduced colony formation[33] to increased differentiation and apoptosis and reduced proliferation.[34]

1.1 Critical Appraisal and Future Works

Spheroids are considered a model of intermediate complexity between standard two dimensional cell culture *in vitro* and tumour growth *in vivo* in terms of complexity. Due to their structure, the cells inside are at the mercy of different diffusion gradients of oxygen and nutrients, just as normal tumour cells would be. The production of extracellular matrix by the cells within the MCTS only furthers to improve the suitability of the model.[35] Like solid tumours, MCTS develop subpopulations of quiescent and necrotic cells in the centre which are similar to regions observed in tumours that are inadequately supplied by the local vasculature. They enable the study of different cell sub populations within the same MCTS and the effects of the different microenvironments' upon them. The cells found within the MCTS also have similar growth kinetics to tumours grown *in vivo*. Similar maximal diffusion distances were also seen in the spheroids as have been recorded in clinical tumour tissue.[36] Essentially MCTS enable the characterization of various sub populations of cells with a variety of microenvironments which could lead to new ways of targeting these populations by taking advantages of the molecular differences or by normalizing these differences.

Isolation of different populations of cells from within the MCTS enabled the individual study and characterisation of these populations *in vitro*. Populations which have been found to be distinct from each other with regards to the proteome in general,[37] and specifically cyclin dependant kinase inhibitors.[38] However much work has looked into MCTS as a model without taking advantage of the differing populations, instead combining them all together and masking any potential variations.

This body of work has established there are biological differences in cells found capable of surviving in areas of necrosis. Furthermore through identification of possible survival mechanism and functional differences, and the proteomic investigation of these cells, possible targets for further investigation have been established. As yet the reliance of the cells upon these aberrantly expressed proteins is not understood, but in order to take this work further research would need to be carried out to validate these targets. The proteomic analysis provided a starting point for further investigation into identifying markers for these viable cells within necrotic tissue. Further work could include validating many numbers of these proteins in order to determine their expression in situ (within the necrotic core of MCTS). Specifically assays to measure active enzyme activity would be imperative in order to determine whether the proteins highlighted in the proteomic experiment had functional activity within the cells in the necrotic core. Specifically investigating the proteins which are linked to the functional biological data presented in Chapters 1, 2 and 3 such as proteins involves in migration and metastasis, chemoresistance and cell survival. Focussing further work on the differences between NCC1 and NCC3 may also be useful. Both were found to be more resistant to 5-FU than the parental cell line yet only NCC1 was found to have a slower rate of proliferation. By investigating this further a drug resistance mechanism independent of the relationship between proliferation and drug sensitivity may be uncovered.

Modelling these processes in MCTS has many advantages; however the next stage of research would need to be concerned with moving from *in vitro* to *in vivo.* Isolating cells from within necrotic regions in xenografts and then clinical tissue would serve to provide proof of principle for using MCTS as an *in vitro* model for this area of research. Furthermore understanding whether this phenomenon is seen only in colorectal cancer or in other cancer types would be important future work.

1.2 Concluding Statement

The aim of this body of research was to examine the necrotic core of MCTS, characterise the cells it comprised of and ultimately determine the existence of any viable cells with biological and therapeutic significance. The final conclusions to be made from this body of work are that within the necrotic core, there is a subpopulation of cells that remain viable. Once returned to favourable growth conditions, they retain the ability to grow and form colonies. These clones have different characteristics to the parental HT-29 cell line and this includes attenuated cellular proliferation, increased chemosensitivity and migration and stem-like characteristics. The biological implications of these findings could be profound as these studies have identified a subset of cells that are traditionally ignored but have the ability to reform tumours once growth conditions become favourable. Therapeutically, some of these cells have a resistant phenotype and although the mechanism of resistance isn't known, it could represent a novel route to generate the resistant phenotype that typically leads to treatment failure in patients. Taken together, the results presented in this thesis provide a novel hypothesis to explain the reason why the extent of tumour necrosis described by several studies is a poor prognostic factor. Tumour necrosis contributes to poor prognosis for patients by providing a microenvironmental niche for the survival of non proliferating metabolically adapted aggressive cells which once conditions becomes favourable again could be capable of reforming a more aggressive secondary tumour. Understanding the biology and exploring the potential mechanisms identified in this thesis could lead to novel targets for therapeutic intervention.

2 References

[1] Fisher ER, Anderson S, Redmond C, Fisher B. Pathologic findings from the National Surgical Adjuvant Breast Project protocol B-06. 10-year pathologic and clinical prognostic discriminants. *Cancer.* 1993;71(8):2507-14.

[2] Martens K, Meyners T, Rades D, Tronnier V, Bonsanto MM, Petersen D, Dunst J, Dellas K. The prognostic value of tumor necrosis in patients undergoing stereotactic radiosurgery of brain metastases. *Radiat Oncol*. 2013;8(1):162.

[3] Hiraoka N, Ino Y, Sekine S, Tsuda H, Shimada K, Kosuge T, Zavada J, Yoshida M, Yamada K, Koyama T, Kanai Y. Tumour necrosis is a postoperative prognostic marker for pancreatic cancer patients with a high interobserver reproducibility in histological evaluation. *Br J Cancer*. 2010;103(7):1057-65.

[4] Edwards JG, Swinson DE, Jones JL, Muller S, Waller DA, O'Byrne KJ. Tumor necrosis correlates with angiogenesis and is a predictor of poor prognosis in malignant mesothelioma. *Chest*. 2003;124(5):1916-23.

[5] Pichler M, Hutterer GC, Chromecki TF, Jesche J, Kampel-Kettner K, Rehak P, Pummer K, Zigeuner R. Histologic tumor necrosis is an independent prognostic indicator for clear cell and papillary renal cell carcinoma. *Am J Clin Pathol.* 2012;137(2):283-9.

[6] Vakkila J, Lotze MT. Inflammation and necrosis promote tumour growth. *Nat Rev Immunol*. 2004;4(8):641-8.

[7] Lin SP, Lee YT, Wang JY, Miller SA, Chiou SH, Hung MC, Hung SC. Survival of cancer stem cells under hypoxia and serum depletion via decrease in PP2A activity and activation of p38-MAPKAPK2-Hsp27. *PLoS One*. 2012;7(11):e49605.

[8] Weichselbaum RR, Dahlberg W, Beckett M, Karrison T, Miller D, Clark J, Ervin TJ. Radiation-resistant and repair-proficient human tumor cells may be associated with radiotherapy failure in head- and neck-cancer patients. *Proc Natl Acad Sci U S A.* 1986;83(8):2684-8.

[9] Deacon J, Peckham MJ, Steel GG. The radioresponsiveness of human tumours and the initial slope of the cell survival curve. *Radiother Oncol.* 1984 ;2(4):317-23.

[10] Fertil B, Malaise EP. Inherent cellular radiosensitivity as a basic concept for human tumor radiotherapy. *Int J Radiat Oncol Biol Phys.* 1981;7(5):621-9.

[11] Ling V, Chambers AF, Harris JF, Hill RP. Quantitative genetic analysis of tumor progression. *Cancer Metastasis Rev*. 1985;4(2):173-92.

[12] Kreso A, O'Brien CA, van Galen P, Gan OI, Notta F, Brown AM, Ng K, Ma J, Wienholds E, Dunant C, Pollett A, Gallinger S, McPherson J, Mullighan CG, Shibata D, Dick JE. Variable clonal repopulation dynamics influence chemotherapy response in colorectal cancer. *Science*. 2013;339(6119):543-8.

[13] Marusyk A, Almendro V, Polyak K. Intra-tumour heterogeneity: a looking glass for cancer? *Nat Rev Cancer*. 2012;12(5):323-34.

[14] Kaern M, Elston TC, Blake WJ, Collins JJ. Stochasticity in gene expression: from theories to phenotypes. *Nat Rev Genet*. 2005;6(6):451-64.

[15] Flynn DF, Mirotchnick N, Jain M, Palmer MI, Naeem S.Functional and phylogenetic diversity as predictors of biodiversity--ecosystem-function relationships. *Ecology*. 2011;92(8):1573-81.

[16] Loreau M, Naeem S, Inchausti P, Bengtsson J, Grime JP, Hector A, Hooper DU, Huston MA, Raffaelli D, Schmid B, Tilman D, Wardle DA. Biodiversity and ecosystem functioning: current knowledge and future challenges. *Science*. 2001;294(5543):804-8.

[17] Fraser HB, Hirsh AE, Giaever G, Kumm J, Eisen MB. Noise minimization in eukaryotic gene expression. *PLoS Biol*. 2004;2(6):e137.

[18] Raser JM, O'Shea EK. Control of stochasticity in eukaryotic gene expression. *Science.* 2004;304(5678):1811-4.

[19] Lewis K. Persister cells, dormancy and infectious disease. *Nat Rev Microbiol*. 2007;5(1):48-56.

[20] Kreso A, O'Brien CA, van Galen P, Gan OI, Notta F, Brown AM, Ng K, Ma J, Wienholds E, Dunant C, Pollett A, Gallinger S, McPherson J, Mullighan CG, Shibata D, Dick JE. Variable clonal repopulation dynamics influence chemotherapy response in colorectal cancer. *Science*. 2013;339(6119):543-8.

[21] Wu WK, Coffelt SB, Cho CH, Wang XJ, Lee CW, Chan FK, Yu J, Sung JJ. The autophagic paradox in cancer therapy. *Oncogene*. 2012;31(8):939-53.

[22] Sui X, Chen R, Wang Z, Huang Z, Kong N, Zhang M, Han W, Lou F, Yang J, Zhang Q, Wang X, He C, Pan H. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. *Cell Death Dis*. 2013;4:e838.

[23] Song YJ, Zhang SS, Guo XL, Sun K, Han ZP, Li R, Zhao QD, Deng WJ, Xie XQ, Zhang JW, Wu MC, Wei LX. Autophagy contributes to the survival of CD133+ liver cancer stem cells in the hypoxic and nutrient-deprived tumor microenvironment. *Cancer Lett*. 2013;339(1):70-81.

[24] Rausch V, Liu L, Apel A, Rettig T, Gladkich J, Labsch S, Kallifatidis G, Kaczorowski A, Groth A, Gross W, Gebhard MM, Schemmer P, Werner J, Salnikov AV, Zentgraf H, Büchler MW, Herr I. Autophagy mediates survival of pancreatic tumour-initiating cells in a hypoxic microenvironment. *J Pathol.* 2012;227(3):325-35.

[25] Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL, Wahl GM. Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res*. 2006;66(19):9339-44.

[26] Locke M, Heywood M, Fawell S, Mackenzie IC. Retention of intrinsic stem cell hierarchies in carcinoma-derived cell lines. *Cancer Res.* 2005;65(19):8944-50.

[27] Bortolomai I, Canevari S, Facetti I, De Cecco L, Castellano G, Zacchetti A, Alison MR, Miotti S. Tumor initiating cells: development and critical characterization of a model derived from the A431 carcinoma cell line forming spheres in suspension. *Cell Cycle*. 2010;9(6):1194-206.

[28] Dörr JR, Yu Y, Milanovic M, Beuster G, Zasada C, Däbritz JH, Lisec J, Lenze D, Gerhardt A, Schleicher K, Kratzat S, Purfürst B, Walenta S, Mueller-Klieser W, Gräler M, Hummel M, Keller U, Buck AK, Dörken B, Willmitzer L, Reimann M, Kempa S, Lee S, Schmitt CA. Synthetic lethal metabolic targeting of cellular senescence in cancer therapy. *Nature*. 2013;501(7467):421-5.

[29] Sutherland RM. Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science.* 1988;240(4849):177-84.

[30] Warrell RP Jr, Frankel SR, Miller WH Jr, Scheinberg DA, Itri LM, Hittelman WN, Vyas R, Andreeff M, Tafuri A, Jakubowski A. Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-trans-retinoic acid). *N Engl J Med*. 1991;324(20):1385-93.

[31] Shin DM, Glisson BS, Khuri FR, Clifford JL, Clayman G, Benner SE, Forastiere AA, Ginsberg L, Liu D, Lee JJ, Myers J, Goepfert H, Lotan R, Hong WK, Lippman SM. Phase II and biologic study of interferon alfa, retinoic acid, and cisplatin in advanced squamous skin cancer. *J Clin Oncol*. 2002;20(2):364-70.

[32] Meyskens FL Jr, Salmon SE. Inhibition of human melanoma colony formation by retinoids. *Cancer Res*. 1979;39(10):4055-7.

[33] Meyskens FL Jr, Alberts DS, Salmon SE. Effect of 13-cis-retinoic acid and 4-hydroxyphenyl-all-trans-retinamide on human tumor colony formation in soft agar. *Int J Cancer*. 1983;32(3):295-9.

[34] Dragnev KH, Petty WJ, Dmitrovsky E. Retinoid targets in cancer therapy and chemoprevention. *Cancer Biol Ther*. 2003;2(4):S150-6.

[35] Nederman T, Norling B, Glimelius B, Carlsson J, Brunk U. Demonstration of an extracellular matrix in multicellular tumor spheroids. *Cancer Res*. 1984;44(7):3090-7.

[36] Sutherland RM. Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science.* 1988;240(4849):177-84.

[37] McMahon KM, Volpato M, Chi HY, Musiwaro P, Poterlowicz K, Peng Y, Scally AJ, Patterson LH, Phillips RM, Sutton CW. Characterization of changes in the proteome in different regions of 3D multicell tumor spheroids. *J Proteome Res*. 2012;11(5):2863-75.

[38] LaRue KE, Khalil M, Freyer JP. Microenvironmental regulation of proliferation in multicellular spheroids is mediated through differential expression of cyclin-dependent kinase inhibitors. *Cancer Res*. 2004;64(5):1621-31.